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Proceedings of the Seminar on Biological Monitoring and Its Use in the NPDES (National Pollutant Discharge Elimination System) Permit Program Held at Chicago, Illinois on Oct 2, 1979

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Proceedings of the Seminar on Biological Monitoring and Its Use in the NPDES Permit Program

Held at the
Conrad Hilton Hotel
Chicago IL
October 2, 1979

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This seminar brought together representatives from industry, state and local governments and biological monitoring consulting firms to present the U.S. Environmental Protection Agency, Region V perspective on biomonitoring requirements for the National Pollutant Discharge Elimination System (NPDES) permit activities.

A limited number of industries have the potential for the discharge of toxicants and they will be required to conduct special testing, monitoring and evaluation of their effluents by utilizing biological testing techniques. This seminar was held to clarify the methods and uses of biomonitoring and its application to setting limits in NPDES permits. Presentations were made concerning biological monitoring phytoplanktons, zooplanktons, macroinvertebrates, fish, bacteria, etc. The range of tests include static and flow-through bioassays, including tests for bioaccumulation, Ames tests for mutagenicity, and some rapid assessment methods such as the fish cough response test.

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Chicago, Illinois
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PREFACE

From the earliest federal water pollution control legislation, the concept of protection of human health and the environment has been important in the development of regulations to control waste discharges into the waters of the United States. The Clean Water Act of 1977 reemphasized this concept by requiring as a paramount concern the control of toxicant discharges which may cause damage to human health or the environment. The U.S. EPA and the state and other pollution control agencies are responding by increasing their efforts to work with industries and municipalities to identify, evaluate, and control the discharge of toxicants. Such endeavors during the past few years have resulted in the installation of industrial and municipal treatment systems providing major control of conventional pollutants and, directly or coincidentally, some toxic substances as well.

Nationally, U.S. EPA is developing effluent guidelines and water quality criteria to control the discharge of a select number of toxicants of national concern. In addition, U.S. EPA's regional offices and the states are now working to implement programs to address facilities which may be of specific local or regional concern because of their particular products, processes, raw materials, etc., or to address the unique assemblage of industries in a given river or lake basin.

Two of the tools being utilized to evaluate the potential for toxicant discharge are biomonitoring and industrial process evaluations. A limited number of industries having the potential for the discharge of toxicants have been or will be required to conduct special testing, monitoring, and evaluations utilizing these tools. The information obtained from these assessments, along with national guidelines, is being utilized in the NPDES permit reissuance process to evaluate the need for additional toxicant controls.

Although many industries, the states, and the U.S. EPA have done some biomonitoring, the need for increased use of biomonitoring as a tool to control toxicants has become increasingly evident. Therefore, this seminar was held to clarify the methods and uses of biomonitoring and its application to setting limits in NPDES permits. Presentations were made concerning biological monitoring tests which make use of freshwater and marine biological systems - phytoplanktons, zooplanktons, macroinvertebrates, fish, bacteria, etc. - in tests for toxicity in process and waste discharges. The range of tests include static and flow-through bioassays, including tests for bioaccumulation, Ames tests which use bacteria to test for mutagenicity, and some rapid assessment methods such as the fish cough response test.

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WELCOMING REMARKS

John McGuire
Regional Administrator
U.S. EPA, Region V
Chicago, Illinois

I would like to welcome all of you to the seminar on Biological Monitoring and its use in the NPDES Permit Program. Your participation is, of course, the key to the seminar. The turnout - some 400 people - suggests that this seminar is needed and that many of you are aware of the importance of biomonitoring in developing limitations for toxic substances in NPDES permits.

Although many industries, the states, and the U.S. EPA have done some biomonitoring, the need for increased use of biomonitoring as a tool to control toxicants has become increasingly evident. We thought it would be useful, therefore, to hold a seminar to clarify the methods and uses of biomonitoring and its application to setting limits in NPDES permits. Notices of the seminar were sent to those industries in Region V which we believed would benefit most from biomonitoring or which might be required to do so.

While the concept of protecting aquatic life was paramount in the earlier Federal Water Pollution Control Legislation, more recent legislation, the Clean Water Act, has emphasized the control of toxic substances with emphasis on the aquatic environment and human health. We in EPA, the states, and other pollution control agencies have responded by increasing our efforts to work with industries and municipalities to surface, evaluate and, where necessary, control the discharge of toxicants. Industries and municipalities throughout the country have installed treatment systems which have provided major control of the more common pollutants and coincidentally some toxic and persistent chemicals.

Nationally, EPA is developing effluent limitations and water quality criteria to control the discharge of a select number of toxicants of possible national concern. Region V, along with the states, is now working to implement a program to address those other toxicants which may be of local or regional concern because of unique concentrations of industries. Biomonitoring and industrial process evaluations are some of the tools being utilized to evaluate the potential for toxicant discharge. A limited number of industries are or will be required to conduct special testing, monitoring and evaluations utilizing these tools. This information along with the national guidelines will be utilized in the NPDES permit reissuance process in evaluating the need for additional toxic substances control.

In working with industries to date, a number of common questions have been raised. The purpose of this workshop is, in part, to answer these questions and to let you know some of our thinking concerning biomonitoring, process evaluation, and the need to place greater emphasis on the control of toxic substances.

I am particularly concerned that efforts to control persistent substances be given an extremely high priority in the Great Lakes Basin. I need not recount here the list of these substances which have been found thus far. We appear to be finding others, also. As U.S. Cochairman of the Great Lakes Water Quality Board I am charged with the responsibility for implementing the Water Quality Agreement between the United States and Canada.

That agreement calls for increased attention to and vigilance in the control of toxic and persistent substances in the Great Lakes Basin. While we have made and continue to make progress in that area we are obligated to increase our efforts and to ultimately control these dischargers. The fact that the Great Lakes also suffer from atmospheric input of many contaminants only increases the need to reduce or eliminate those dischargers which are most controllable. While there is an absolute need for heavy emphasis on the Great Lakes, we are also aware that significant efforts are needed in our other major basins such as the Ohio River Basin. To a lesser extent, efforts are also needed in the Mississippi River Basin.

Biomonitoring represents only one tool for achieving our goals. As Dr. Mount will discuss later, biomonitoring has not traditionally been used for measuring persistence. Perhaps it is time we break with tradition and as appropriate, use all of our tools - biomonitoring, process evaluation and others - more fully.

Again, I want to welcome you here today to this Biomonitoring Seminar. I would like now to turn the program over to one of EPA's most distinguished scientists, Dr. Donald Mount.

BIOMONITORING OF EFFLUENTS IN PERSPECTIVE

Donald I. Mount
U.S. EPA, Environmental Research Laboratory
Duluth, Minnesota

I'm not exactly sure of the backgrounds of most of you, and I don't know how much you know about biomonitoring - maybe you know a lot more than I do. Biomonitoring is a term that means many things to many people and it is misunderstood because of that. As John said, the thrust of biomonitoring really relates principally to the protection of organisms, including man, and I'd like to use a definition or a concept of biomonitoring in a very restrictive way this morning as I make my comments so that you'll know what I'm talking about.

There are many kinds of biomonitoring, including those in which we do field studies to assess the health of a stream or a lake in relation to an outfall. But I think the upstream-downstream studies of the fifties are probably not useful in most heavily industrialized or urbanized locations simply because there are so many outfalls in close proximity that one cannot really tell the effects of one outfall from another. And so, although it has a place in terms of receiving water quality, I don't really see that as being especially pertinent to the problems that I think we are here to talk about today, which are problems related to the toxic characteristics of particular effluents. So, in my comments, I'm going to be talking more about laboratory or out-of-stream monitoring as opposed to in-stream monitoring, although I certainly don't want to rule out the use of cages in waste streams and similar techniques which might be considered in-place monitoring.

I made a statement in the meeting in Washington on the first of August which I thought was fairly obvious, but I find, upon discussion with other people since then, it was not really understood. There is really only one way to determine the toxicity of anything - and that's with an organism. I don't believe there is any other way to decide what toxicity is or how toxic something may be without the use of organisms. I think by definition it's only a response that organisms give to an exposure. There is no electronic instrument that I know of that measures toxicity directly, although certainly measurements can be correlated in many instances with toxicity measures.

An important implication is that there is no true reference standard for toxicity. There is no way to know precisely the exact toxicity of some particular chemical - and I'm talking now about pure chemicals as well as effluents. This means that the approach to toxicity measurements must be different than when you measure the amount of mercury in a sample and know precisely what you have in your standard. How do you know which toxicity measurement is right and which one is wrong? They are really almost relative to each other. And maybe everyone is exactly correct for the conditions under which it was done - but nonetheless you will get different answers. So, it is inconceivable to me that any discharger who is concerned about the impact of his effluent on receiving water or any regulatory agency who is concerned about the control of what we have rather sloppily been calling "toxics", wouldn't use organisms to determine toxicity because there is no other way to measure it. If we would use organisms, we would eliminate the problems of relationships between the response of organisms and the chemical measurements made with electronic measurements.

The most important reason we have effluent control or pollution control, be it air, water or whatever, is for the protection of biological organisms somewhere, including ourselves, of course. There are a few uses of water which may not be directly biologically related, but for the most part, they are. It's obvious then that the role of organisms to measure toxicity of effluents is certainly appropriate and far overdue. If you go back in the literature - Tarzwell, Doudoroff, and others - were calling for biomonitoring in the late forties and early fifties. Only just now are we becoming aware of the value that it has.

Now, let me insert one other word of caution. None of us are proposing that biomonitoring is a replacement for chemical measurements - that's just clearly not the case. Both are needed - they are both tools that tell us different things about the effluent. And in some cases chemical analysis may be far better than biomonitoring for what we want to know. But I think that if you will stop for a moment you will realize that the kinds of chemicals that we are now focusing our attention upon are not BOD and suspended solids but complex organic chemicals. To measure those chemicals is a real job. If you think that the cost of purchasing the electronic equipment to make these measurements is bad, even though it may be $1/2 million in some instances, the cost of buying competent chemists who can operate that equipment and who know what it's telling them is an even greater cost. The capital cost of the equiment is small compared to the manpower to operate an installation like that. And so, we certainly do need to have cheaper and more effective methods to use as routine tools and save those costly analyses for places where we really need them. So, if we talk in terms, as some speakers later today may, of $450 or $500 for a biomonitoring test, that cost pales when compared to the alternate costs if we have to go the analytical route. I remind you again that we are not now talking about measuring BOD, which is a cheap thing to do. We are talking about much more difficult measurements for a great many more chemicals. That's not to say there won't be surrogate measures to which we can relate toxicity as we learn more about it, but at the present state-of-the-art, that doesn't seem to be possible.

Some of the advantages of biomonitoring, as opposed to only chemical monitoring, are that it probably more closely approximates the receiving water conditions than nearly anything we can do, and it does consider the interactions that may occur between the components of a waste stream. Again, for the most part, I'm talking about complex wastes that are made up of many things and vary in quantities. This is the difference between working with industrial wastes and classical toxicology. I'm not considering in these comments biomonitoring for human health. There is another line of defense which makes that concern a little less important in that few of us drink untreated water. The protection of the organisms does not have that second line of defense. So our discussion more commonly, and maybe rightly, is focused on aquatic organisms rather than on the human health aspect even though I think we all agree that the health aspect is more important.

While there may be additive or synergistic properties in wastes there are also antagonistic properties or subtractive properties, whatever you want to call them. From this standpoint we get at the problem of mixtures very effectively - probably as effectively as any way we can approach the question at the present time, because the state of toxicology in the aquatic area, and I think also in the mammalian area, is not really advanced far enough yet to know how to deal with interactions. You can measure the interaction of one mixture but there's no way to relate that information to another mixture. I suggest as proof of this, you ask your physician what he knows about the interaction of commonly used drugs in your body and you'll find out that the state-of-the-art is embryonic.

A third distinct advantage is that we are measuring the target response and not an index of it. When you measure COD, TOC, or mercury in a waste, you are not measuring the same condition as exists in the receiving water. You come much closer in a biomonitoring test.

Let me digress to comment on how animals behave in pickle jars. There may be differences between the way animals respond in glass bottles and the way they respond in the stream, but the alternative is to do it all in the stream. If any of you have done much in the way of in-stream work, you know the tremendous cost that's involved and the tremendous frustration you often feel. It may grate against your scientific credibility to think about extrapolating responses of animals in bottles to real-life situations. But there is a two-edged sword which I'm afraid industry, in particular, is not aware of. If we can't extrapolate with effluents, then when registering a new chemical under TOSCA, I don't see where EPA has any other choice but to say that you can't extrapolate those either. That means testing lake trout, shovel-head catfish, and elephants. My point is, either we extrapolate or we grind to a halt country-wide. I don't know what position the agency's going to take - I don't make policy in the agency - but if they ask me, I'm going to say, if we can't extrapolate on effluent biomonitoring, then we can't extrapolate on the registration of new chemicals either under FIFRA or under TOSCA. There's going to be some error, but we all work with error daily. More often than not, the difference in response of animals in the bottle is not because they're responding differently than in the stream; we are just not aware of all the conditions to which they are exposed. If you look at the costs of biomonitoring and compare them to the probable costs of chemical analysis, biomonitoring is an efficient use of resources.

There are obviously disadvantages. The world's problems are not going to be solved by a simple 96-hour or 24-hour bioassay. Some disadvantages are that there will be physical-chemical changes in the properties of the wastes after they are released into the receiving water. If we run tests with the whole effluent, even though we may use receiving water as a diluent, we are not going to measure all of the same chemical and physical interactions that go on in a stream. But that is equally true of BOD measurements. It's a difficult and rather subjective art, I think you'll all have to agree, to get from a BOD test to the DO in a stream. And we have a very comparable problem when we work with whole effluents. But nevertheless, you can certainly gain a good first cut at what may be of concern in your waste by use of biomonitoring.

It's difficult to measure chronic effects, whether they be chronic toxicity, carcinogenicity, or bioconcentration, without going to rather extensive and longer-term tests. At the present time, we simply don't have any overnight test that looks all that promising to get at the chronic effects. So, you cannot expect to go from a simple short effluent test to a no-effect concentration or a safe concentration in the receiving water, and have any credibility. We are not trying to go from an effluent to a safe receiving water concentration with a simple lethality test. It's just nonsense to think you can do that as yet.

As John mentioned earlier in his comments, most of our biomonitoring to date has not attempted to measure persistence. If there is one property about chemicals on which we ought to focus, a property which we ought to eliminate above others, it is persistence in the environment. If you think of previous headlines in the newspapers of the last ten years and identify the chemicals named - methyl-mercury, DDT, Dieldrin, PCB's, Mirex, Kepone - they all have that property in common - they are environmentally persistent. If I were making new chemicals or concerned about the effects of my discharge on receiving water, I would clearly want to know what the persistence of this material is. If we did not have persistent chemicals in discharges, all of our toxicity or environmental contamination problems would be local problems - they would not be ubiquitous like we have had in the past. We are not as adept at effectively measuring persistence with simple tests. I think we are much better at measuring toxicity, but measuring persistence is doable.

There are special considerations in using effluent tests. They cannot be done only once. Effluents are variable from day to day as all who have worked with effluents know. Such toxicity measurements are only as good as the representativeness of the sample. If the sample is not typical of the waste, then neither is the toxicity, and you cannot say much about the impact on the receiving water. Biomonitoring tests must be cheap enough, simple enough, and short enough, so that they can be done on enough samples to characterize the waste. What I have said is equally true of chemical measurements as well. Measuring the TOC in a waste once a month is not telling us much for many wastes. There is no sense in making the toxicity measurement any better than the representativeness of the sample used.

One message I hope to leave with you from this discussion this morning is that it's time we consider toxicity as a property of wastes which may need to be reduced and which we should measure. This does not necessarily mean that effluents must have zero toxicity. Toxicity is relative and we are concerned about those where the exposure that will occur in the receiving water is harmful.

DISCUSSION

Question: I know very little about the testing that you are speaking of - this biological monitoring - but if we do have to put this into industry, in what form do you think it will be? Will this be a fish pond, or are there any different types of scientists or chemists that it involves? What does it mean? What will we have to resolve - the topics here at issue?

Answer: In general terms without getting into specifics of tests which we are going to do later today, it's clear that tests must be simple, doable by something other than the PhD, and they must have a reasonable cost. I think if we don't achieve those things in the effluent tests, we have achieved very little. I think Sandy and perhaps some of the other speakers may be in a better position to know what form that takes. We've been talking in terms of tests that can be done offsite in most instances.

Question: Would EPA consider it good enough to hire a free-lance company out to do the biomonitoring? In Syracuse there is a company that just runs bioassays, and we send a lot of our samples there.

Answer: Again, I can't speak for the agency, but those of us in the agency who have been talking about these problems I think all recognize the essential roles which consulting firms or consulting laboratories must play in this program. I think whether a discharger goes to a consultant or does it himself will depend in large part on the size of his operation, the kind of staff that he has, and the particular location. But I don't think any of us at this point are ruling out the use of consulting firms.

THE NPDES PERMIT POLICY AS IT RELATES TO BIOMONITORING

Sandra Gardebring
Director, Enforcement Division
U. S. EPA, Region V
Chicago, Illinois

Good morning. I would like to add my personal welcome to that of John McGuire, the Regional Administrator, and to thank you for your enthusiastic response to our seminar on biomonitoring. The fact that so many of you are here underscores the timeliness of this topic and the value of biomonitoring as a tool in achieving clean water.

What are we really talking about when we say biomonitoring? As used in this conference, biomonitoring, or biological monitoring tests are studies which make use of freshwater or marine biological systems - phytoplankton, zooplankton, macroinvertebrates, fish - and in some cases bacteria - to test for toxicity in process and waste discharges. The range of tests includes static and flow-through bioassays, Ames tests, and some rapid assessment methods such as fish cough response. Depending on which test is used, the data generated can indicate acute or chronic toxicity, carcinogenicity, mutagenicity, or can suggest potentially problematic bioaccumulation of organic chemicals, trace metals, or other toxicants.

However, it was the passage of the 1972 Act, and its requirement of a nationally uniform water discharge permitting program with technology based standards, which finally focused federal and state resources on water pollution clean up. In this region, we issued about 7,800 industrial permits with BPT or water-quality-based requirements, addressing a wide range of water pollutants. Emphasis was often on the more common pollutants such as suspended solids, BOD, and pH, along with more persistent toxicants such as heavy metals and PCBs. The long process of treatment design and installation was started, and although some dischargers had to be "persuaded" through federal and state enforcement actions, overall, our efforts at achieving compliance in this region have been quite successful. Some 75 percent of industrial dischargers in our six states now meet the limitations of their NPDES permits, and the remainder of the major permittees, almost without exception, are the subject of state or federal enofrcement actions aimed at bringing about their compliance.

During this same period, several other things were going on which brought us to where we are today. First, the instrument makers and our friends in the laboratories combined to bring us tools and techniques which allow the discovery and quantification of water pollutants at lower and lower levels - parts per million, parts per billion, parts per trillion. At the same time, we weren't very good at telling whether the presence of such pollutants - even so-called "exotics" - at these very low levels was significant or not. The "safe" levels in ambient air or water just weren't known for many pollutants.

Secondly, both the public and those of us whose business is pollution control became more and more aware of the real risks associated with the unregulated flow of these chemically complex materials into our environment. PBBs, PCBs, Mirex, Silvex, Dioxin, Kepone - terms known heretofore primarily to white-coated men in chemistry labs - became, almost literally, household words. The removal of solids and the increase in dissolved oxygen in our waterways was a truly remarkable and wonderful effort, but it clearly was not enough when chemically complicated materials with unknown biological effects continued to be discharged.

The state and federal environmental regulatory agencies began to respond under existing laws; a process which was accelerated under the terms of a consent decree agreed to by EPA and the Natural Resources Defense Council in 1976. That agreement, which ended an NRDC lawsuit related to toxic effluents, required EPA to test for the presence of 129 specific toxicants in waste water discharges from 34 industrial categories and to control any of these priority pollutants found at harmful levels via toxic pollutant effluent guidelines regulations. Certain of the technology based limitations and water quality criteria needed to carry out the terms of the decree have begun to appear this year in the Federal Register.

However, we simply cannot afford to wait for or depend solely upon the effort to develop categorical limitations for the 129 pollutants to solve our toxic pollutant problems.

Given that there are nearly 50,000 chemical compounds in commercial usage; given that more than 12,000 of them are suspected toxicants, 1,500 possible carcinogens, and many others considered capable of injuring human health and aquatic biota; and given that we know little or nothing about the synergistic effects of these compounds at even low levels, we must begin addressing the specific problems in our states, whether or not they are related to the 129 consent decree toxicants.

It is in this effort that biomonitoring, when combined with process evaluation and chemical analysis, can be a potent tool both for discovering and for limiting the discharge of toxic substances, and that is how we intend to use it.

Let me provide a few more details about our approach.

EPA authority for requiring biomonitoring stems from Section 308 of the Clean Water Act. The states, in order to receive NPDES authority, are required to have similar authority. Our review indicates that all of the states in Region V do have similar authority and can require biomonitoring and process evaluation either as part of a permit or as a separate independent information-gathering requirement.

Perhaps some of the most important questions, particularly to industry, are: Who are the target industries? Who should monitor? And beyond that, how will the results be interpreted and used? As I have noted previously, biomonitoring is only a tool and as such it must be applied with full knowledge of its limitations. It can be relatively expensive. The results are sometimes less than clear and the tests often must be repeated.

On the other hand, biomonitoring is useful in telling us the response of an organism to a complex effluent. The interaction - simple additive effects and synergisms - of chemicals in an effluent and the ultimate effects upon aquatic life are difficult to judge from available literature and criteria. Many, if not most of the bioassays used to develop criteria are run with one, perhaps two, or rarely three substances interacting. Information on substances which bioaccumulate is even more limited and various biomonitoring tests can be used to derive data to address these toxicant effects.

I am sure that during the seminar you will explore many more of the limitations and benefits of biomonitoring. I have highlighted only a few of those just to indicate our own awareness of the need for reasonable application of biomonitoring and all other tools.

Returning to those questions I posed previously: Who will be required to biomonitor? How will they be selected?

Let me outline generally our thinking. Since most of the state and federal interest is focused on major dischargers, those lists provide a natural place to start. There are between 500 and 600 industries on the major list in Region V. Those industries which might well be required to biomonitor include chemical, pharmaceutical, pesticide, plastics, iron and steel, rubber, chlor-alkali, paper, and other similar chemical-type facilities.

In part, the level of treatment may also play a part in our decision. Certainly a process stream receiving treatment with carbon filtration will be looked upon differently than one receiving more conventional treatment. An industry discharging only noncontact cooling waters with total process stream recycle or deep-well injection may also be viewed differently.

Furthermore, I want to make it clear that we do not intend to ignore those dichargers which have been previously classified as minor. We believe that there are more than a few of those dichargers which may contain significant contaminants and require closer scrutiny than they have received in the past. Again, a careful evaluation of the facility, the process involved, present treatment and the likelihood of a toxicant being in the discharge will be necessary before biomonitoring is required.

Finally, discharges to the Great Lakes or its tributaries will receive emphasis for finding and eliminating or severely limiting those substances which bioaccumulate and may pose a long term hazard to other aquatic life or higher animals including man. Our agreement with the Canadians, and the general tendency of bioaccumulative substances to be particularly troublesome in the Great Lakes, forces us to place more emphasis in that area. However, our emphasis on the Great Lakes will not be to the exclusion of other major drainages such as the Ohio and Mississippi Rivers and their tributaries.

It should be obvious that the need for biomonitoring will largely be determined on a case-by-case basis. Once the determination is made that biomonitoring is needed, then the specific test or series of tests must also be determined. Much will depend upon the nature of the contaminants or suspected contaminants. In some cases, "failure" in one test may lead to another. In a few cases where data presently exists, long term bioaccumulation may be required directly. We estimate at present that 15 to 20 industries may need to conduct long term bioaccumulation tests. It is also estimated that 150 to 200 industries will require other forms of biomonitoring.

How then will the results of these tests be applied? How will permit limitations be developed from test results? Let me again outline our general approach. At the outset, I should note that state water quality standards generally require that nothing be toxic within the mixing zone. Some states specify that the concentration of a substance within the mixing zone shall not exceed the 96-hour TLM for that substance. We interpret these requirements to mean that the discharge at the end of the pipe must not be toxic. In addition, state water quality standards require that outside the mixing zone concentrations of substances must not exceed 1/10 of the 96-hour TLM. These standards will be used to develop permit limits for appropriate toxicants. In some cases the toxicant will not cause a problem at the discharge or exceed 1/10 of the 96-hour limit at the edge of the mixing zone, but it may bioaccumulate in aquatic organisms or be a known carcinogen, mutagen, or teratogen. For some of these toxicants we will have the information necessary to develop appropriate control levels for protecting aquatic organisms and human health. For others, very little will be known and we will have to work with the states and the industry to develop appropriate control levels based on data derived from biomonitoring, process evaluations, and toxicity reduction plans.

At this point I would like to address those of you in the audience who represent industry. Much of what I have said so far has been addressed more to the state people - engineers, biologists, lawyers - those often intimately involved in the NPDES permit program. In some cases the states are ahead of us in the biomonitoring area. In other states little if any biomonitoring has occurred. In addition to exchanging information with our states, part of the purpose of this seminar is to spread ideas and information and to let you know what we are thinking about. For those of you who may be most affected by what we are saying and proposing here, we believe it is to your advantage not to wait. We believe it is to your advantage to seize the initiative before the states or EPA knock at your door.

Looking early at your facility gives you all the options, including one of the most important elements - time. With no regulatory onus you are free to investigate and experiment. You are able to select the most cost-effective approach and time with due regard to plant operation and least interference with union rules, worker scheduling, and the other constraints which generally translate to time and money. Nothing is less cost-effective and more disruptive than when the regulatory agency imposes a tight schedule on an industry because of concerns generated by a process totally outside of your control. You also should utilize biomonitoring in evaluating which treatment scheme you would install to control conventional or other toxic substances so that you can obtain the greatest incidental control of toxicants.

For instance, we are just beginning a program to collect samples from selected river basins for a broad organic scan of fish flesh to detect substances which may have bioaccumulated. These scans tell us whether or not something of concern has been or is being discharged. Where something is found, for example, hexachlorobenzene (HCB), chlordane, PCBs or any of dozens of other compounds, we will then begin to look for probable sources. Knowing the nature of the various industries in a basin we can usually pinpoint likely sources or a probable source. This will lead to inquiries to the industry such as a 308 letter requiring process evaluation, biomonitoring or additional chemical sampling. Those of you who receive such inquiries may not have a problem but it's pretty certain that your company will feel some anxiety and there will be time constraints for your response.

In such an instance, you can no longer plan the most optimum and least costly path - you're under the gun. To avoid that situation you need to begin now to do some of these things on your own. What are your processes? What chemicals go into them? What by-products are produced? What are the trace contaminants? If one process seems to pose a problem, should you have a bioassay run or check for bioaccumulation? What process modifications can you make to reduce or eliminate the problem as opposed to end-of-pipe treatment? We are not asking you to do everything at once. We are asking that you establish priorities, putting a high priority on toxic substances, particularly those which are persistent, which bioaccumulate or which represent a human health hazard. By taking the initiative you will be able to establish priorities and work within the many other constraints. In the meantime, we are encouraging the states to initiate fish flesh monitoring, biomonitoring, process evaluation, and other tools of the pollution control business.

If any of these lead to your industry you will be ahead of the game and probably save money if you've already done some of these things on your own schedule. It should be obvious that the direction we have outlined is for a long term - we do not expect biomonitoring or process evaluation to suddenly appear in all NPDES permits. However, as these permits are reissued this year and in 1980-81 we will be stressing these areas and they will begin to appear more often in permits.

In summary, the tools we are discussing today are not new, but they will receive increased use in the future particularly as they enable us to identify and control toxic substances. We are hopeful that this seminar will provide greater understanding of where we are headed: increased cooperation between state and federal agencies and industry; greater interplay between biologists, engineers, and administrators of agencies and companies; and finally greater understanding and use of biomonitoring as a tool. These in turn, are, of course, only steps along the way to our ultimate goal: fishable and swimmable waters in all parts of this country.

DISCUSSION

Question: I am wondering if biomonitoring will be acceptable to the 129 priority pollutants.

Answer #1: (Mount) If one runs bioassays on an effluent, recognizing that an effluent is a complex mixture, the 129 priority pollutant assessments will never be satisfied. I don't think I could really answer that question for certain, but as much as I understand the consent decree, I would think that biomonitoring would not be acceptable to the 129 priority pollutants. It might be useful, but it seems to me that the consent decree requires certain levels of treatment; I don't think that toxicity is one of the parameters by which that treatment is devised.

Answer #2: (Gardebring) I think that answer is generally correct. I would not want to say, however, that biomonitoring will not be part of the process that we will use when dealing with those particular parameters. We are all kind of feeling our way along on the consent decree pollutants, and I think there will be some use of biomonitoring in that process. However, it won't be solely a toxicity-based approach.

Question: Along those lines, in our experience, we have found that we are exceeding a level of toxicity for priority pollutants, but in our biomonitoring program there is no toxicity. What can you do about the inconsistency there?

Answer: (Mount) I don't think that it's an inconsistency. I mentioned this morning that there will be antagonisms in the effluents as well as the opposite, which is what we always hear about - the synergistic properties. I think that one ought to try to separate biomonitoring from the specific consent decree program because they arose out of different contexts even though they are generally both aimed at controlling toxicity. We have all felt, for a long time, that when you put mixtures together different things happen than when you keep them separate, which is why I think biomonitoring effluents is so valuable. It gives us a better handle on the real world impact in those situations.

Question: What if we do scan the river basins and collect data by biomonitoring on our effluents although these are not on your consent decrees. When it comes down to the actual permit process and legal proceedings, all of the data which we collect before the process begins becomes evidence both in our favor and in many cases against us - am I correct?

Answer: (Gardebring) I think that's generally correct. All I can suggest to you, sir, is that your alternative is simply to wait, as I indicated, until you get a 308 letter. The 308 letter will have a time schedule in it, but if you think you have a problem with some contaminant or other toxic material that you can identify in your effluent, you're better off to begin now. I wouldn't suggest that you charge out and hire the consulting firm this lady mentioned earlier, but I would suggest that you might want to come in and sit down with the state officials or people from my staff and talk about the kinds of things that should be looked at.

Question: What do you anticipate your reaction will be, from an enforcement point of view, if a toxicant which wasn't listed in the permittee's application or control in the permit shows up through the biomonitoring?

Answer: (Gardebring) I think it's a little hard to answer that question in the abstract. The purpose of the program that we have outlined here is to identify dischargers that have toxic materials present in their effluents. Recognizing the limitations that are suggested through the use of this word, I think we all generally know what we're talking about. If we identify the presence of a discharge that has that kind of material through biomonitoring, we'll ultimately be looking to some kind of permit limitation. It may be a negotiated limitation; it may be one based on a legal process that you're painfully familiar with, but we will, I suspect, in at least some of those cases, be looking to permit limitations based on the presence of that material.

Question: What happens to these laboratory tests - are these data subject to subpoena?

Answer: (Gardebring) I presume that they are. I know no reason why they would not be, although, as I said, I am more of an administrator than a lawyer. I can't imagine that they would not be subject to subpoena unless they are subject to some kind of confidentiality claim through one of the provisions of the Act.

Question: What if there is a natural toxicant in the waters coming into our plants and that through our waste treatment systems it's not removed - are we responsible for that?

Answer #1: (Mount) Well, I think it's a legal question, not a biological one. However, I think that there should not, in general, be any problem in measuring the increased toxicity, if there is any which your effluent has as a result of natural toxicants, by appropriate biomonitoring tests.

Answer #2: (Gardebring) There is a specific answer to that question. I know that the Act deals with that very specifically within the regulations and unfortunately it has escaped me. I will try to get someone to get the answer for you. (See statement by Glen Pratt, p. 44 -- Ed.).

Answer #3: (Mount) I don't really see that the problem is any different, in principle, from other situations where particular chemicals or substances, especially metals, are more toxic in one type of receiving water than in another. I think it's the same situation as when there are properties of receiving waters which are not good for the organisms and therefore affect the tests done in those waters.

Answer #4: (Gardebring) I would just like to comment on an earlier question asked by this gentleman referring to whether or not biomonitoring would replace screening for the 129. It is our intent, in the June 14 draft-collated permit application performance regulations, not to use biomonitoring as a screen for the 129 in some cases. We do, however, encourage that in certain situations the permit writer include biomonitoring as part of the recorded departments in developing the permit limitations. I just wanted to make that clear and that the NRDC consent decree is not necessarily the driving force behind the indigenous biomonitoring program. It was just something that got us started, and now that my office in Washington is in the process of posing the consolidated permit application form regulations as well as what we call the biomonitoring policy protocol, biomonitoring can be used on a case-by-case basis in addition to greater chemical test condition.

Question: What is the status of application factors these days? The laboratory fish production indices and all these other things that your lab has been involved with. Is it basically a 96-hour median tolerance limit or is an artificial number chosen in order to give safe dosages? Is there any flexibility in this procedure? What's the present-day status? Is it going to be 1/10, 1/100, or what?

Answer: (Mount) Well, I'm not sure, unless you have an hour, that I can answer that question. I think it's fair to say that if you do not have long-term toxicity data on an effluent or on a chemical, that is, all you have is some estimate of legality from a short test, there isn't any better way to predict the no-effect level than with the application factor. Now having said that, I should also say that there is a lot of error in doing it this way. As a result, there will probably be some error in any prediction. Short of having data, I think it's about the only tool that I know of right now. Based on comments that we received on the guidelines for the consent decree criteria documents, we are now thinking along those lines mainly because one of the early comments that came back consistently was that you cannot generalize among chemicals. We agree with this. We are accepting that this is the situation, and we are faced with this question: You have chemical X and you have only 96-hour lethal data on it. What is the estimated no-effect level? We believe that the application factor is eminently better than guessing. I want to quibble with some of your terminology. It is not a safety factor; I think I heard you mention the word safe or something like that, but maybe I'm misquoting you. In any event, the application factor, as it is being proposed, is an attempt to get from the lethal level to the no-effect level, and I think that's different from a safety factor. We do know that one can put chemicals in categories in terms of the magnitude and the application factor, for example, some are on the order of 1/10 or less, some are on the order of 1/1000 or more, and others fall in between. So there are some generalized differences that you can see among chemicals, but when you try to decide whether the application factor is 1/12 or 1/11 or 1/15, you find that the data are simply not good enough to allow you to do that kind of precise work. How much of that is due to experimental error, problems of techniques and variations between laboratories, and how much is real? We simply don't know.

Question: We've heard that we're going to have to run chemical analyses for specific toxicants, and, based on your comments, if we're always within limits we should never see any negative response of the biotest for that parameter. Isn't the biotest kind of redundant on that?

Answer: (Mount) The question was, if one is within the chemical limits as measured by analytical chemistry, one would not expect to see toxicity in a biomonitoring test, and the answer to that is no. I think you will expect to see it from time to time, and we do. The reasons are, first of all, that the limitations are clearly not on all chemicals in any consent decree. And the second point is that we have not yet come to grips in the consent decree work or in any other work - all of these standards deal really with the problem of nature.

Question: Let's just assume that you are operating a waste treatment plant for industry. You have biomonitoring set up, and you determine that your effluent is toxic; you don't necessarily know what to attribute it to. What's your next step to correct the problem, since that's what we're interested in doing?

Answer: (Mount) I think chemical analysis is the next step, along with more toxicity measurements, and I think the way in which most people go about it, if there are multiple streams making up the effluent streams, is to go back up the line and see where the toxicity is coming from - where most of it is coming from. I think it is far cheaper to locate the source with biomonitoring and make the identity with chemical analysis, than to try to do it with chemicals all the way. If you go the analytical route, you may measure 475 chemicals before you find the one that's causing the problem. But if toxicity is your concern then it seems obvious that toxicity ought to be the endpoint in the measurements that you make. Ultimately, of course, you'll have to identify it analytically.

Question: Isn't it possible to get a toxic result and not actually have a toxic condition? What if there is not enough DO in your effluent stream, or another condition exists that is not necessarily favorable to the organisms that you are using?

Answer: (Mount) I think what you're referring to is the situation where the conditions of the test are different from the conditions of the receiving water. In that case, I think the test would be considered improper.

Question: If all of these companies and all of these laboratories that are government run get into bioassay, and we choose something like rhododendron roots or something, isn't there going to be a logistic shortage of test vehicles and equipment? How long will it be before you have enough supply to meet the demand where you think this is profitable?

Answer: (Mount) I don't think it's a problem because I think that the profit motives of those who do the test are such that they will stay well ahead of the rate at which the regulatory agencies can implement this. If that is not the case then I think it's obvious, and common sense dictates, that we have to move at a pace so that we can keep up with the demand. But, you will be surprised at how many firms already have this capability, and the evidence for this is the fact that the price is coming down. Now it may not be in absolute dollars but it certainly is in real dollars. There was a time when a static bioassay was $800, now they're considerably cheaper in a number of places. So I really think that that's not a big problem. We're not talking about sophisticated equipment at this stage nor expensive equipment compared to the modern analytical chemistry lab. I think the problems in measuring 129 chemicals will be more costly than this.

Question: You know that the chlorination process causes carcinogens, and I am wondering if your biomonitoring program will take this into consideration, maybe change, modify, or ban the chlorination process?

Answer #1: (Mount) I know of no specific program addressed particularly to the chlorine problem. I am aware of the controversy in the regulatory as well as the scientific community. I suppose that some of the bioassays that will be run may identify chlorine as a particular problem and then we would deal with it at the particular site. I don't know that any of the biomonitoring that we have been talking about here, which is really intended to be fairly site-specific, would go to any sort of national policy or program change with regard to chlorination. I think that issue exists in the agency, in state agencies, and in Canada, and is the subject of a lot of conversation. There has been some change in the policy and there may indeed be more, but at this point I know of no particular biomonitoring effort.

Answer #2: (Gardebring) I know of a number of places in the country where they have gone to dechlorination in order to avoid the problems of the toxicity of chlorine itself. Whether chlorine itself is a problem as far as aquatic toxicity is concerned would depend on how much time elapses before you measure that toxicity, and that's why the comments I've made about persistence are particularly important. But with regard to the problem of forming other chemicals that may be carcinogens or other such properties, I think it's recognized that there are some that are formed in certain kinds of situations. It seems to me that it's going to be a negotiation between the trade-offs, which are what we gain by chlorinating and what we lose by chlorinating. It is simply a conflict of not being able to do everything perfectly; there has to be a trade-off. I think that we may come to recognize, in our society as a whole and in this field as a whole, that there are worse villians running around than coliforms.

Question: When we think of other large industrial nations, where do they stand on the subject of biomonitoring and its application to regulations? Can you give us some information on that? Are we the leaders, or are we the followers? Are we in step with the rest of the world?

Answer #1: (Mount) I can't give you any information. I can't say anything about the requirements of regulatory agencies in other countries. But I do know that there is much activity in Europe, for example, in England and South Africa, in developing biomonitoring tests.

Answer #2: (Gardebring) The only place I know of now is over in the United Kingdom on the Thames where they've instituted continuous monitoring have had systems going for the past about 6-10 years or so - very active.

Question: What is the perceivable time frame as far as post regulations?

Answer: (Gardebring) In terms of our biomonitoring requirements, I can't give you any specific number. Certainly as we go into the next round of permit issuance and as we look toward BAT, we will be starting down that road. Within Region V, as I said, we are starting to target particular geographic areas where problems may exist. We are doing that through the fish flesh analyses and other data gathering that we have. So, it will be, if not in the immediately forseeable future, within the next year, into the calendar years 80 and 81, as we go through the next round of permit issuance. It's coming very soon.

Question: Do you envision biomonitoring for POTW's eventually?

Answer #1: (Gardebring) I guess Glen (Pratt) should probably answer this question because he's the pretreatment expert, but let me give you my own personal perspective. I think that that really depends on the extent to which we are able to put into effect an effective pretreatment program. We know that there are a tremendous number of toxic materials that pass through normal POTW systems and get out into the waterways. I think the way to go about it is to look into some process evaluations for industries that discharge in the public systems, and I would say generally for this round at least, we will be looking principally at industrial dischargers. For municipal dischargers, our focus will be on getting the pretreatment program going.

Answer #2: (Pratt) Let me briefly add to that. There might be a few cases where we would be talking about having the municipality do biomonitoring on its influent rather than on its effluent. It would be used as part of a program to go back and control specific industries or to look for industries that might need control. I know we are looking, in several cases, at having biomonitoring requirements for specific industries that would be going into municipal systems. In general, to emphasize what Sandy said, we are going to be looking in our normal reissuance process so that when a given permit comes up for reissue, particularly in the short-term permits, we're going to be requiring biomonitoring as part of the permitting process, as well as where we have become aware of specific problems within a given watershed. So I think it's going to be through both of those channels. As far as the question on which countries are doing things, I think that we should probably look even closer to home. There are significant differences between the regions and the states. Our region appears a little bit behind several of the other regions. And certainly, some states have been more able to use this in their regulatory process. So you will see variations in biomonitoring program applications over the next year or two. This is just a matter of getting a program going with a lot of people involved.

Question: Are you looking at static testing as adequate?

Answer: (Gardebring) It's not the total answer, but we're certainly contemplating using it. I think we'll hear more about that as the day wears on.

STATIC TEST USING FISH

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My topic this morning is on static bioassay using fish, and I know my attempts that follow in describing static bioassays will be unfamiliar to some of you and will be very old hat to others. To those to whom it is going to be a little bit new, all I can say is, well, welcome to the mystical world of bioassay. I say that with some facetiousness because we frequently run into the response that it is a very mysterious type of test, and sometimes we, as biologists, have some difficulty in accepting that response. I think that if you can accept a test standard which is accepted by many people and is a very useful tool, then we don't have any trouble accepting the static bioassay test.

The static test is a nondynamic test consisting simply of taking a solution of a mixture, placing test organisms in it, and leaving it for a specified period of time to observe the response of the test organisms. Normally the mixture is not added to or taken away from during the test, and once the test is set up it is left alone for the test period. There is another type of static test that is called a static "renewal" test wherein, on a specified periodic basis, a solution of the test concentration is added to or exchanged with the original solution. There are several advantages and disadvantages to the static test. The advantages meet many of the criteria that we were into this morning. That is, it's simple, it's cheap, and although it requires trained technicians, it doesn't necessarily require the highly trained PhD biologist. The disadvantages, of course, are that in the static test, the toxicity that might be present may change during the test period due to either degradation or volatility of the substance, absorption of the toxicant, uptake of the toxicant by the organisms, or several other factors. The flow-through test that you will hear about later takes away those disadvantages. However, the basic experimental design and basic requirements are well known for static tests, and I will refer you to many excellent references. They are all very similar and describe the tests in detail. These are: Standard Methods, ASTM, and the EPA documents on effluent toxicity testing. ORSANCO has a bioassay procedure and, of course, there are many state agency documents describing static testing.

When we are considering the performance of a static bioassay perhaps it's worthwhile to understand what our objective is in looking at effluents. It is simply this: The basic purpose of the effort of biomonitoring at the point of discharge is to determine if acute toxicity to aquatic organisms exists as a property of that discharge and if it does, then there may be a potential impact in the receiving water. The next step would be to try to evaluate that potential impact. In attempting to determine this objective (which might be termed as a screening test) the static test serves well in measuring this unknown property of effluent testing.

I think the best way I might be able to describe the static test, before I refer to some of the requirements that are in all the standard references, is to discuss some of the types of questions that we have received during the performance of our program in Region III. We have been going on-site to industrial and municipal treatment facilities and running some flow-through and static tests. I think it might be good to repeat some of the questions that we frequently run into from the industrial personnel. Once we get by the question: Why are you there? (and I always refer to the statement that I just made earlier, i.e., we are simply trying to determine the acute toxicity of that discharge), the next question that we ordinarily get is: "What is the test organism that you're using in the static bioassay and why is it the fathead minnow? Why don't you use some indigenous form from the receiving screen that's out there from our discharge? Why don't you use carp or blue gill, or anything else? Basically we have a real problem with trying to use wild stock as the test organism. You don't know anything about the quality of the group of fish that you might collect, and you have a very difficult time getting a constant size and age. You don't know the history of the organism, or what it has been exposed to, and you do not have control over the disease problems that might be inherent with the strain. There is a whole host of variabilities that may play a role in evaluating the results of the test, in addition to the difficulties of capturing the critters in sufficient numbers to begin with. Therefore it's much better for the test organism to be a fish that can be cultured, can be obtained commercially, and that can be maintained in healthy stocks. We are trying to reduce all the known variables, so the only variable that we would be measuring is the quality of the effluent. By maintaining the tight control over the test organism that is used, you have eliminated another area that could be distractive to the results of your test. As to why the fathead minnow, it can be said that it is native to the midwest and eastern North America, and it is the white rat, so to speak, of the aquatic testing world. There are a lot of data available for the toxic response of this organism. It can be cultured in the lab, and we have been able to use it successfully. There are other organisms that probably are just as sufficient. Other fish such as blue gill and channel catfish all meet the same kind of requirements, and in some situations where there is a cold water environment, the trout meet these criteria, also.

The next question that we run into frequently is: "We are going to use the fathead minnow; where do we get the fathead minnow?" In our region, we are attempting to develop a list of commercial sources for fathead minnow, and, upon request, we would supply that list to anyone who is attempting to set up a bioassay program. We can't guarantee the quality of the fishes that you want from these sources. We have, in our program, attempted to make use of these commercial sources as well as other sources that we have available to us, i.e., federal and state hatcheries. We have had some problems in utilizing the commercial sources, and we have had some excellent results, too. The point is: The fathead minnow is available, and the person or industry who receives the fathead minnows must take extra precautions to be sure that the quality of the fishes is well maintained. Another question that we frequently get is: How are you sure they are fatheads? This brings a chuckle from us too, because we didn't really expect that question. Just recently we had a couple of industries bring that one up to us. My only response at this time is: If you feel there may be some question about the credibility of the source, that is, the species that you are getting from a commercial source, I would simply recommend some local taxonomist to look at a sample of the fish, make identifications, and provide the industry with a statement of certification that these are indeed fathead minnows. As this bioassay monitoring program goes down the road, regulatory agencies will have the opportunity to visit industry or consulting labs that are performing toxicity tests, and we will have the chance to make identifications ourselves.

Another question that comes up - maybe this one comes first - is: How much is this going to cost? You've heard some comments this morning about some cost figures, and the guides that we have received from various contacts range somewhere between \$300 and \$500 for a static test. And that means the sample shipped to a central laboratory where the static bioassay is performed.

I would like to move on to some of the basic requirements in performing a static bioassay. What we are trying to emphasize, and no doubt in some cases it will be overemphasized, is the quality control necessary to have a good bioassay program. The reason that we need to emphasize this, I think, is to reduce attempts by either a regulatory agency or a discharger who is having bioassay performed to discredit the quality of data that is produced. We are concerned with several areas of controls in the test. All of these are described in the references that I mentioned earlier, and I simply want to review them in this discussion today. We start off with facilities and equipment, and one of the primary things that is recommended, of course, is to have an adequate temperature control so you are reducing that variable which can influence the results. You should have a well-ventilated facility. It should be shielded from outside disturbances. In that regard, so you know what I'm talking about here, it has been suggested in certain tests that disturbances, such as laboratory people walking by highly visible test containers, noise from machinery and other apparatus, and so forth, can affect fish behavior and the test results. I think this is probably overemphasized, but we should try to control that condition. There may be very specific additional facilities required for maintaining a stock of fish for a long period of time. For instance, you may need to have apparatus for continuous flow to your stock of fish in order to keep them healthy. You may need a UV sterilizer for disease control or recirculatory system for treating the water with charcoal filters. The materials that you use for water transport, the construction material of the containers that you hold the fish in during either the test or during holding times should be relatively inert.

It has been recommended that 316 stainless steel, glass, teflon, or a similar type of substance would minimize leaching, dissolution, or adsorption. Another frequent item that's discussed is the size of the containers. This is dependent upon the size of the test organisms and the loading specifications, which are adequately described in the references. We do not want to have undue consumption of oxygen in the test container; therefore you need a certain volume of the test solution depending upon the size of the organism.

Another area that's important to consider and one that needs to have particular attention paid to it, if you're running frequent and intermittent tests, is the cleaning procedure that must be performed between the static tests that are run. The cleaning procedure, as simple as it may sound, needs to be specified. Again, we're trying to reduce all of these variables down to where the only variable is the quality of the effluent. The cleaning procedure recommended is a simple washing with a detergent, rinse, use of a solvent to remove any organics that might remain from the previous test, dilute acid wash to remove reside metals from the previous test, and then adequate rinsing.

When conducting the static test, normally more than one dilution or concentration is set up, and then the question arises: Where do you get your dilution water? Dilution water is acceptable if the healthy test organisms survive in it throughout an acclimation period, that is, a period of acclimatizing your fish to the dilution water prior to test, and if they survive in that dilution water during the toxicity test without showing signs of stress such as discoloration or unusual behavior. For effluent toxicity testing, the dilution water should be a representative sample of the receiving water and should be obtained from a point as close as possible to but upstream of and outside of the zone influenced by the effluent. It may be practical to transport batches of water in tanks to the testing site if the dilution water immediately upstream of your discharges is not acceptable.

Pretreatment of the dilution water should be limited to a basic filtration through a nylon sieve having 2 mm or larger holes to remove debris or to break up large floating or suspended solids. The water should be obtained from the receiving water as close as possible to the time the test starts. It should not be obtained more than 96 hours prior to testing. If unacceptable, or if acceptable dilution water cannot be obtained from the receiving water, some other uncontaminated, well-aerated surface or ground water commercially available can be used. The water should have a total hardness, total alkalinity, and specific conductance within 25 percent, and pH within 0.2 units of the receiving water at the time of testing. If a substitute dilution water cannot be obtained, reconstituted water may be prepared and used for the dilutant.

Many of the items that I have mentioned about the loading of test organisms for example, are described in the reference books on static bioassay. I refer you again to Standard Methods and the EPA manual. The loading and static tests and test chambers should not exceed 0.8 g/l at temperatures of 20°C or less and not exceed 0.4 g/l at temperatures above 20°C.

There's always a question about dissolved oxygen. If you must aerate to maintain a minimum level, and this does happen occasionally, the test solution should not be permitted to fall below 40 percent saturation for warm water species, and 60 pecent saturation for cold water species. If you do aerate, then the exact methodology that you use should be detailed in a report. Of course, organisms are not to be fed during the tests and there should be some accompanying chemical and physical data of the solutions that go with your report.

In summary, items that should go in your report describing the static bioassay include the following:

- The name of the test method
- Investigator and laboratory and the date the test was conducted
- A detailed description of the effluent including its source, date, and time of collection, composition, physical and chemical properties, and variability
- The source of the dilution water, the date and time of its collection, its chemical characteristics and description of pretreatment
- Detailed information about the test organisms, including scientific name, length and weight, age, lifestage, source, history, observed diseases, treatments, and acclimation procedure used
- Detailed description of the test procedure, test chambers, including the depth and volume of the solution, the day the test was begun, the number of organisms per treatment, and the loading
- The definition of the adverse effect that you measured for the test - was the data for immobility or whatever?
- A summary of general observations of other effects
- The number and percentage of organisms in each test chamber including the control chambers that died or showed the effect used to measure the toxicity
- How the LC50 was calculated should be described, and the EPA may want the recommended procedure described in detail and examples given
- Along with this should be any other relevant information that applies to the static bioassay.

STATIC TEST USING ALGAE

William Miller
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Corvallis, Oregon

I am going to present a static test procedure that has been developed by the Corvallis Environmental Research Laboratory. This test procedure is an ecological test procedure that can be used to define a toxic response to various compounds whether they be a single metal, a complex waste, or any combination of interacting pollutants that enter the aquatic environment. I would like to describe the development of this test from the basic foundation to the complex application that we use today.

The test procedure was initiated in 1969 by a joint government industry task force on eutrophication. The test was developed primarily as a tool to define the interaction of nutrients in an aquatic environment and to find out what effect might be obtained if you could regulate the nutrients entering an ecosystem.

The key requirements that we adopted when we were developing the algal assay test were:

- It should be designed so that technician-level personnel can operate it
- The equipment and instrumentation should be modest and readily obtainable. This is one area in which we may have failed (since an electronic particle counter is necessary to conduct the test accurately within an efficient time frame)
- It should be standardized to give reproducible results, and the geographic location should not affect test results
- All results should be applied with judgement to real-world conditions.

The test organisms that we chose for our algal assay test was Selenastrum capricornutum. It is a green unicellular alga that is easily cultured in the laboratory and is amenable to electronic particle counting. The electronic particle counter was incorporated to measure cell density as well as mean cell volume changes within the cells during the growth period. The combination of the cell density measurements and the mean cell volume changes can then be used in a calibration curve corresponding to dry weight, which is the final product used in the test evaluation.

Two basic publications, developed in 1971, which form the foundation of current assay research resulted from our initial efforts: an interlaboratory precision test of the provisional algal assay procedure, and a standardized algal assay static test. (This static test has been further refined (Miller, Greene, and Shirogama, 1973) and is included in Standard Methods (1975) and is also in the ballot process with ASTM.)

One of the next things that we accomplished was to define the effects of nutrients in 49 different lakes throughout the United States. Four basic productivity groups were identified: low, moderate, moderately high, and high productivity. We were able to classify the productivity potential of the 49 lake samples sent to us by limnologists throughout the United States through the use of a laboratory bioassay. In 23 of these lakes where we had a wide background of limnological data, the algal assay test was able to predict the productivity perfectly (Miller, Maloney, and Greene, 1974).

No single chemical test or biological measurement can be used to define all the interactions regulating biological productivity in natural waters. The relationship between algal assay bottle tests and measurements of indigenous phytoplankton in Long Lake, Washington, has been reported (Greene *et al.*, 1976). A high correlation ($r = 0.95$) between both mm^3 indigenous phytoplankton/l and mg chlorophyll a/m^3 ($r = 0.93$) and mg dry weight *S. capricornutum*/l was achieved when consideration was given to whether the reservoir was stratified or homothermal.

The ratio of TSIN to Ortho-P content in test waters can be used as a "guide" to nutrient limitations in natural waters. Waters containing N:P ratios greater than 11:1 may be considered phosphorus limited. Those containing N:P ratios less than 11:1 can be considered nitrogen limited for algal growth. Confirmation of a nitrogen or phosphorus limitation prediction is obtained by analysis of the assay response to singular and combined nutrient (N,P) and/or chelator additions.

In most cases, the trophic status of lakes and impoundments is based on their bioavailable nitrogen and phosphorus content. Those waters containing greater than 0.015 mg bioavailable P/l and 0.165 mg bioavailable N/l are generally eutrophic.

It is important to note that, in the presence of adequate nitrogen with phosphorus concentrations from 6 to 1,860 mg/l, 1 mg/l of available phosphorus will produce 0.43 mg dry weight of our test organism. Also, 1 mg/l of available nitrogen (expressed as the summation of nitrite, nitrate, and ammonia-N) supports 0.038 mg dry weight of our test organism.

Some of our research indicated that the presence of toxicants prevented certain waters from attaing the predicted yield or nutrient limitation status.

Miller, Greene, and Shiroyama (1976b), have reported that the inhibition of specific heavy metals upon the growth of S. capricornutum may be linear (0 to 100 percent) with the increase in zinc content of test waters, but nonlinear for the increase in copper and cadmium content beyond 20 and 40 percent, respectively. These growth responses have established the sensitivity of S. capricornutum to the bioreactive state of these heavy metals. The greater than 95 percent I_{14} algistatic (inhibitory) response of the test alga in these test waters is similar to that of sensitive indigenous species to accidental or recent discharges of heavy metals (an algicidal response is verified when a subculture from an algistatic test water fails to grow in assay medium). However, this inhibited response does not necessarily reflect the growth potential of indigenous algae which have evolved from long-term chronic exposure to heavy metals.

The study of heavy metal interaction in natural waters is complicated by uncertainty of the form, concentration, and biological reactive state of the metal. Thus, with few exceptions, the chemically analyzed heavy metal content of a test water may not reflect the resultant biological interactions and productivity in natural waters. The growth response of S. capricornutum to conditions of heavy metal stress in natural waters is in essence a "biological response model" of complex physical and chemical interactions. The resultant biological response (maximum standing crop) is an integration of the combined effects of solubility, ionic strength, metal concentration, and contact time which regulate toxicity of the heavy metal to the test organism.

The response of the standard laboratory algal test organism to the addition of Na_2 EDTA, alone and in combination with nitrogen and phosphorus, to heavy metal-laden test waters, has been shown to correlate ($r = 0.82$) with indigenous phytoplankton standing crop (Greene et al., 1978). The indigenous phytoplankton growth in these waters can be attributed to: 1) adaptation to their environment; 2) natural decomposition, and/or complexing of the heavy metals by both organic and inorganic ligands; and 3) the presence of adequate nutrients.

A case in point is the Spokane River. The Spokane River received clarified wastewater from the Spokane Treatment Plant which was scheduled to be upgraded to secondary treatment. The City of Spokane needed to determine whether or not they should go to phosphorus removal as a treatment step and what affect this nutrient reduction might have upon downstream productivity, even though the Spokane River is also subject to upstream heavy metal inflow from a smelter near Wallace, Idaho.

We started by defining the response of our laboratory test system to specific heavy metals within the Spokane river system. We found that regardless of the algal species' interaction within a natural system, our laboratory test could define the growth response if the system was nutrient-regulated. However, in the Spokane River, the indigenous organisms had 83 years to develop a tolerance to the heavy metals. Therefore, the regulation that was imposed upon the City of Spokane to actually go to phosphorus (nutrient) removal was indeed justified since the heavy metals in this particular system were not solely responsible for the deterioration of the environment. In other systems we may find that the heavy metals are responsible and therefore we would have to attack the heavy metals entering the system.

How can the algal assay bottle test be used to assess the impact of a complex waste? Since the components responsible for regulating the biological productivity in natural waters are not necessarily identified through chemical analysis, an alternate method is needed. The algal assay bottle test can be used to indicate the presence of bioreactive components or growth regulators within the waste. These components include (but are not limited to) nitrogen, phosphorus, trace elements, heavy metals, herbicides, and pesticides. The significance of the algal test is that both growth stimulation and growth inhibition can be defined.

We evaluated 23 textile waste samples representing eight manufacturing processes by seven assay techniques to define their toxic properties. The bioassessment organisms included freshwater and marine algae, crustacea, fish, and mammals. A comparison of the sensitivity of these bioassays (Table 1) showed that the algal assay bottle test, using S. capricornutum, was one of the most sensitive tests used in the textile waste survey. This test not only identified toxic wastes, but also those that were stimulatory (Shiroyama, et al., in preparation).

It is important to consider the following factors when designing an assay protocol to evaluate the environmental impact of complex wastes:

- The method of entry into the receiving water (i.e., direct discharge after primary, secondary, or advanced waste treatment; percolation through soils; etc.)
- The anticipated final concentration of the complex waste within the receiving water.
- The degree to which the test waters are representative of those receiving the candidate complex wastes.

In conclusion, the algal assay bottle test is a viable tool for the study of nutrient limitation and heavy metal toxicity. It also shows great potential for the evaluation of complex wastes. The validation of the test to define its sensitivity to broad classes of industrial wastes is of prime importance. The biggest stumbling block in this validation is the evaluation of the toxic and/or stimulatory effects of organic compounds. The reasons for this are: 1) the safety factor in handling the compounds; 2) the volatility and insoluble nature of these compounds; 3) the lack of knowledge about the mode of interactions causing the toxicity; and, 4) the expense of chemical identification of both the parent compound and its degradation products within the test system.

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Table 1
COMPARATIVE BIOTEST RESPONSES FOR TEXTILE EFFLUENTS*†

	Freshwater Ecology Series			Recommended Interpretation		Marine Ecology Series		
Textile Plant	Fathead Minnow (96-hr LC_{50}) % Secondary Effluent	Daphnia (48-hr EC_{50}) % Secondary Effluent	Selenastrum (14-day EC_{50}) % Secondary Effluent	Selenastrum 20% Secondary Effluent $\%I_{14}$	Selenastrum 20% Secondary Effluent $\%S_{14}$	Sheepshead Minnow (96-hr LC_{50}) % Secondary Effluent	Grass Shrimp (96-hr LC_{50}) % Secondary Effluent	Algae (96-hr EC_{50}) % Secondary Effluent
A	19.0	9.0	11.3	53	----	62.0	21.2	f
B	NAT[b]	NAT	----	--	83	NAT	NAT	g
C	46.5	41.0	----	--	187	69.5	12.8	90
D	NAT	NAT	----	--	100	f	f	f
E	NAT	7.8	< 2.0	95[e]	----	NAT	NAT	10 to 50
F	NAT	81.7	----	--	598	NAT	NAT	85
G	64.7	62.4	----	--	390	NAT	NAT	59
H	c	40% dead at 100% concen.	7.8	92	----	f	f	f
J	NAT	NAT	----	--	76	f	f	f
K	NAT	NAT	----	--	57	NAT	NAT	77
L	23.5	28.0	12.0	81	----	NAT	NAT	1.7
M	NAT	60.0	----	--	149	f	f	f
N	48.8	100% dead at all dilutions	< 2.0	95[e]	----	47.5	26.3	2.3
P[a]	NAT	NAT	----	--	38	f	f	9.0
R	16.5	8.0	8.8	95	----	f	f	f
S	NAT	NSA[d]	----	--	382	NAT	NAT	g
T	46.5	NAT	----	--	1911	68.0	34.5	70
U	NAT	12.1	----	--	377	NAT	NAT	g
V	36.0	9.4	----	--	232	f	f	94
W	55.2	6.3	1.0	95	----	37.5	19.6	50
X	NAT	NAT	----	--	163	NAT	NAT	g
Y	NAT	NAT	----	--	261	f	f	f
Z	NAT	42.6	15.5	84	----	f	f	f

[a] Sample inadvertently collected prior to settling pond. [b] No acute toxicity. [c] Diseased batch of fish nullified this analysis. [d] No statistical analysis because heavy solids concentration obscured the analysis; the sample did not appear to be acutely toxic. [e] 95% growth inhibition in 2% solution of secondary effluent. [f] Analysis not performed on this sample. [g] Growth inhibition < 50% in 100% solution of secondary effluent. * No chemical mutagen was detected by 10 microbial strains. † No rat mortality after 14 days due to maximum dosage of 10^{-5} m^3/kg body weight (LD_{50}). However, six samples (B, C, F, L, N, and S) showed potential body weight effects, and sample R resulted in eye irritation.

FLOW-THROUGH TEST USING FISH

William Peltier
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Athens, Georgia

I looked at the program today and I noticed I was listed as being located in Duluth, Minnesota, and I know that there are a lot of industrial representatives in attendance today that have plants in Region IV and wish I were in Duluth, Minnesota. This is because Region IV does have an aggressive program, as some of you know, and, hopefully, today I can pass on some of Region IV's experiences regarding the flow-through testing utilizing fish as the test organism.

There are several types of flow-throughs; the first is a continuous flow-through system, and that's exactly what it means. There is a continuous flow of the pollutant mixed with a continuous flow of dilution water that, after mixing together, goes to an aquarium containing fish. This procedure can be accomplished in several ways. One is through the use of metering pumps, where prescribed volumes of a pollutant and dilution water can be delivered to a mixing chamber. Another approach is the use of a constant water level head box of the type that many of you have seen in some of the research laboratories or in some of the state mobile laboratories. This second approach uses capillary tubes of different inside diameters inserted in head boxes containing the pollutant and dilution water which allows for a continuous regulated flow into a mixing chamber. In both examples, following the mixing, the solution flows into the replicate test tanks containing fish. These are two examples of a continuous flow-through system.

The second type of flow-through system is the intermittent flow-through system which is widely used and is the type used in Region IV. The intermittent flow is patterned after the famous Mount and Brungs (1967) proportional diluter system upon which we all have tried to improve over the past years. It's like trying to build a better mouse trap. For instance, Region IV has designed and built a total solenoid system which is described and pictured in the EPA 1978 publication Methods for Measuring the Acute Toxicity of Effluents to Fish and Invertebrates. This system will be shown during the slide presentation and its operation explained to those people that aren't familiar with this diluter system. In this system, flows to the proportional diluter can be adjusted to allow for the filling of the separate dilution water and effluent chambers, and then when they are completely filled, a liquid level switch triggers the emptying cycle. All chambers empty into mixing chambers and from there flow into the aquariums containing the test organisms. There is a delay in the flow of solution going to the aquariums during the filling period. This delay varies anywhere from 2 minutes to 15 minutes and is usually dictated by the desired turnover time in the aquariums.

The flow-through system has long been used in the fish toxicology field. Applications of the system have been in the area of fish, invertebrate, algal, and bacterial testing. At the present time in Region IV, flow-through tests are being used to determine the acute toxicity of effluents on fish species - acute meaning a short-term exposure period of usually 96 hours or less in duration. Flow-through tests also can be used for a longer duration or chronic testing. Chronic testing may include exposure to a pollutant for a portion of the fishes' life or may include the entire life cycle. A life cycle test usually starts with embryos and continues through life stages such as larval, juvenile, adult, and second generation embryo larval stages. Of course this approach is time-consuming and very expensive. In the past, most of the flow-through testing has been conducted using a single chemical as the pollutant and determining its affect on fish. This type of work has been conducted at universities, industries and federal facilities. Flow-through testing has been used to establish safe limits or no effects limits for chemicals such as pesticides, toxaphene, and endrin or the heavy metals, zinc, copper, and mercury.

The use of the flow-through system can be taken a step further and used in multiple chemical testing. For instance, with a slight modification of the diluter system, it has been used to determine the interaction of toxaphene when mixed with malathion. The main thrust in Region IV's toxicity testing program is to determine the toxicity of complex effluents by utilizing the flow-through system. The system is very useful when one is dealing with a "witch's brew" of compounds that are being discharged into the receiving water. The system is very useful in detecting wide variations in effluent concentrations during testing. In Region IV, effluents have been tested where in excess of 60 organic compounds were identified in a single effluent; however, they were not limited in the NPDES permit. At times, fish lethality has occurred well into the test and has been attributed to a slug release of effluent. Fortunately, because a flow-through system was being utilized, these slug releases were detected by the fish dying.

The flow-through system can also be used to determine the bioaccumulation of individual compounds or compounds in effluents by fish when they are exposed over a certain period of time to the pollutant. Fish tissues can then be analyzed for residue accumulation. The test can be taken a step further by sacrificing a portion of the fish for chemical analysis and then flushing the aquariums containing the remaining fish with uncontaminated water for a period of time. Following a period of exposure to the uncontaminated water, the remaining fish are sacrificed and the tissue analyzed for the depuration of the pollutant. Also, the system can be utilized by industries as an on-line monitor to allow the permittee to continually evaluate the effluent for changes which could adversely affect fish in the aquarium and permit the industry to headoff potential fish kills in the receiving water.

Additional uses can be made with slight modifications to the system to study the avoidance of fish to various concentrations of pollutants, the fate of pollutants such as degradation products, or decay rates. As you can see, the flow-through system offers a wide application of uses in the area of aquatic toxicology.

The following are advantages in using flow-through systems. First, in addition to the acute test results, the duration of the exposure period can be lengthened to derive chronic or sublethal results. Second, the problem with maintaining a proper DO level can be reduced considerably in a flow-through system. This problem is not completely eliminated because some effluents have a very high biological oxygen demand or chemical oxygen demand, and because of this, it has been necessary to aerate the solutions in the aquariums in order to maintain suitable oxygen levels. However, for the most part, in flow-through systems, the oxygen level is easily maintained without the aid of aeration. As a result, larger fish can be tested or increased numbers of fish can be tested for each concentration. Third, the metabolic waste products from fish, which deplete the DO, are flushed from the aquariums and do not cause the problems experienced in static tests. Fourth, it is easier to achieve a steady-state chemical concentration in a flow-through system when testing single compounds or mixtures of the compounds. Fifth, in Region IV, approximately 50 to 75 percent of the effluents were variable in their concentrations. As a result, the flow-through system was useful in detecting slug discharges. It's the slug discharges that usually cause the fish kills in our receiving waters.

Additional advantages are better control of the concentrations of the volatile pollutants and better precision.

The following are some of the disadvantages. The first would be that this sytem requires a higher degree of technical expertise to conduct the test. Second, a greater degree of equipment sophistication is required as will be seen from the slide presentation. Third, it requires copious quantities of dilution water. Fourth, at times it can require much more space. Finally, the most important disadvantage is cost. After polling 187 consulting firms located in Region IV during August 1979, it was established that 27 of them had the capability for conducting toxicological studies. Some firms provided information on the cost of various tests. Therefore, some cost estimates are available at this time. An acute flow-through test conducted at the consultant's laboratory on a single compound or an effluent ranges between $700 and $1,000. The lowest estimate was $400 with the highest at $1,500. For conducting acute flow-through tests in the field on an industrial effluent, the consultant's cost ranged from $5,000 to $7,000 for an 8-10 day study. The lowest estimate was $3,000 and the highest, $15,000.

At this time, I would like to show a few slides. The first slide (Figure 1) shows one of Region IV's mobile toxicity trailers set up at an industrial site. In the trailer there are two solenoid flow-through systems. During a test, a portion of the effluent is continually pumped from the discharge ditch into the trailer. Dilution water is collected daily from the receiving water upstream from the discharge outfall. The diluter water is stored in a tank and then pumped into the trailer.

The second slide (Figure 2) shows the proportional diluter solenoid system. The dilution water fills upper chambers; when the last chamber fills, a liquid level switch triggers an electrical mechanism which opens the solenoids, allowing the dilution water to flow into mixing chambers. Similarly, the effluent goes into the small chambers located directly beneath the dilution water chambers. The effluent in the chambers also empties into the mixing chambers at the same time the dilution water is emptying into them.

The third slide (Figure 3) shows what I would call a poor man's diluter system. It is where a minimum of three solenoids are utilized and the operation is based on a vacuum siphon system. The siphon system operates very similarly to the total solenoid system; however, it is much less expensive than the total solenoid system.

At the EPA laboratory in Athens, Region IV is evaluating the Beckman Microtox, which some of you may have read or heard about. I want to make one thing perfectly clear, and that is Region IV is evaluating the instrument for Beckman - we are not endorsing or selling the instrument. For the past 18 months, we have been testing complex effluents to determine the 24-hour LC50s (lethal concentration which is lethal to 50 percent of the test organisms) for fish and daphnids and comparing the results with a 5-minute EC50s (effective concentration which causes an effect on 50 percent of the test organisms) obtained from the Microtox unit. The principle of the test is based on the use of freeze-dried, saltwater, luminescent bacteria which are reconstituted in a buffer solution. An aliquot of cells are then placed in a cuvette which in turn is placed into a chamber in order to measure the light output from the bacteria. Then the effluent is added and the light output is measured again for either a decrease, increase, or no change in the light output. When testing various effluent dilutions, an EC50 can be calculated. The EC50 in this case would be the concentration that would reduce the light by 50 percent. I would just like to briefly say that, from our observations and the limited data, the unit has some advantages and some disadvantages.

The following are the advantages of the Microtox. Its evaluations are very rapid; it takes about an hour to test a control and five effluent concentrations. While the results from the static fish and Daphnia test are not available for 24 hours, the data from the Microtox unit is available in one hour. When using a standard toxicant, the results from the unit are fairly accurate and provide good reproducibility. The unit is very compact and fits nicely on a standard laboratory bench. A direct digital display readout is standard, or a recorder may be attached for continuous recording.

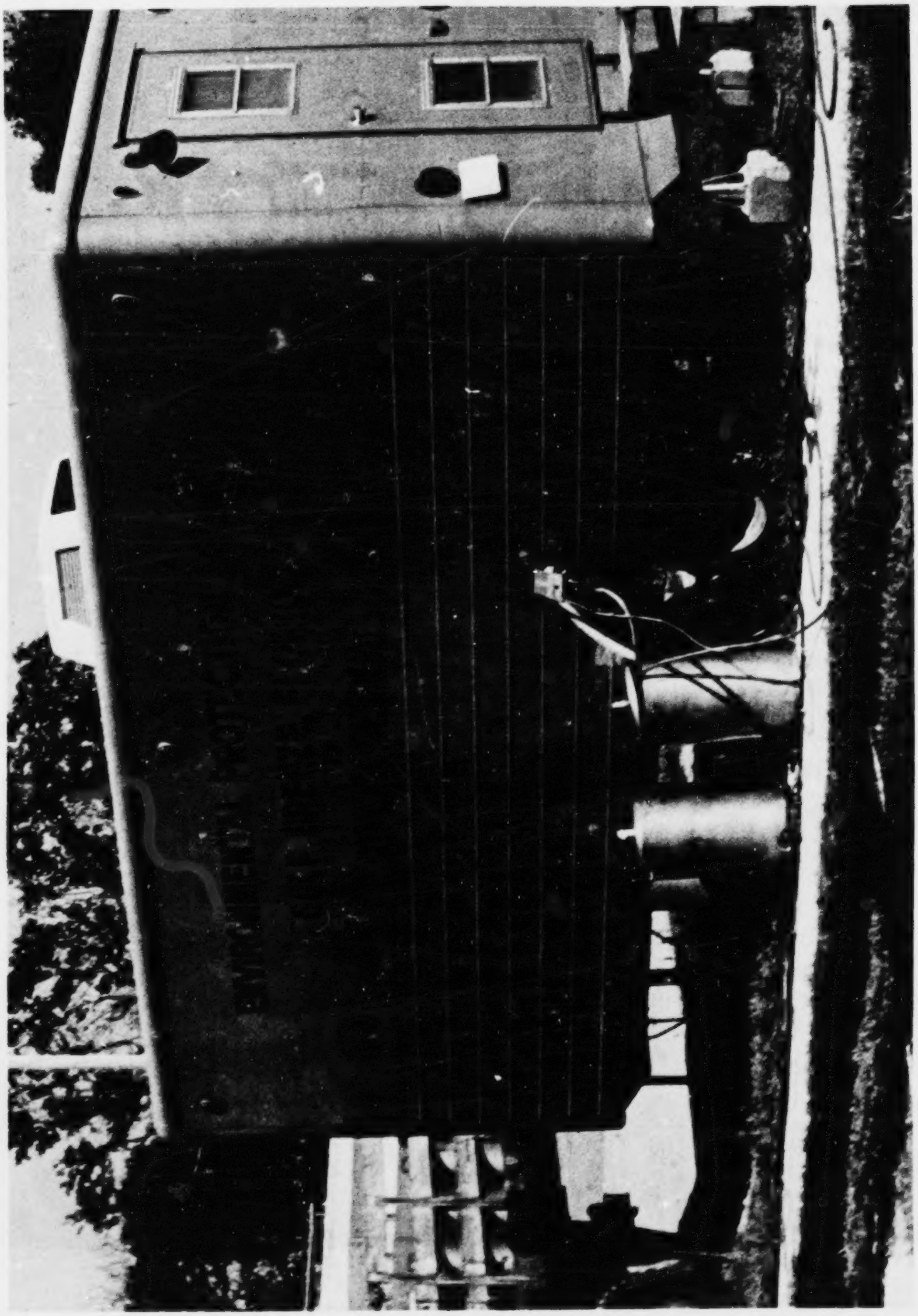

Figure 1. Mobile toxicity trailer set up at an industrial site (Region IV).

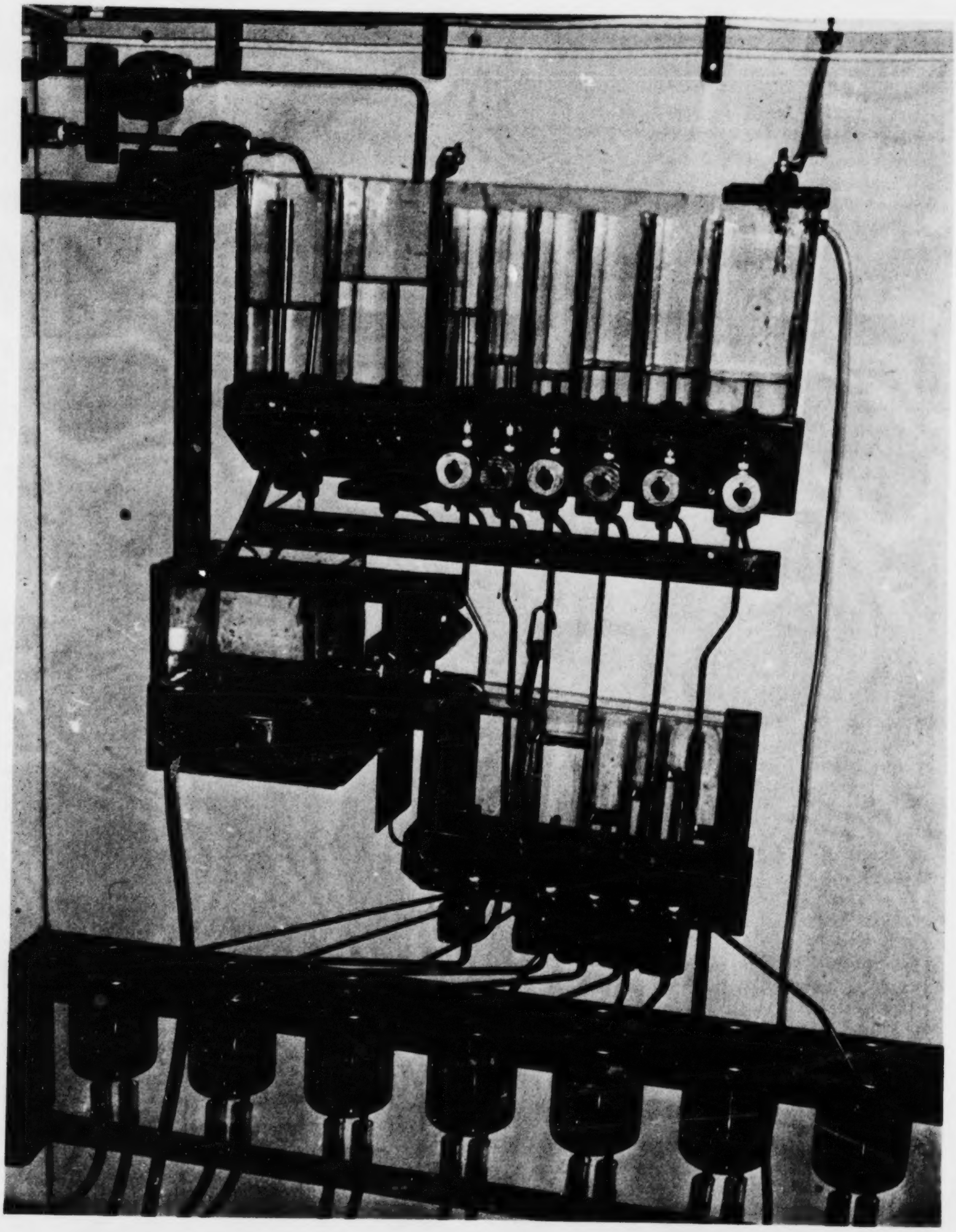

Figure 2. Proportional diluter solenoid system.

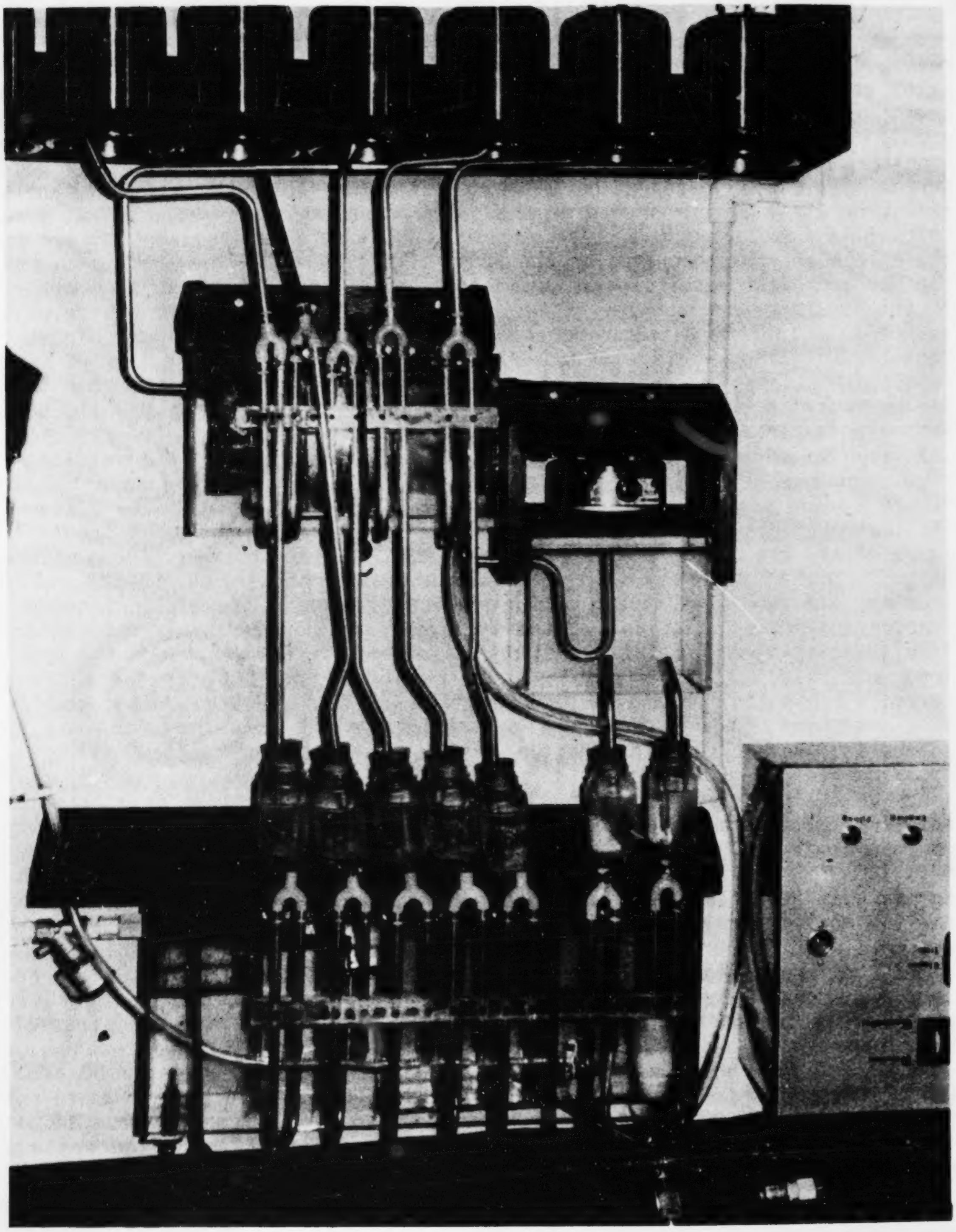

Figure 3. Solenoid, vacuum siphon diluter system.

The following are disadvantages of the Microtox. First, the highest concentration that can be tested at the present time is 50 percent. The reason for this limitation is the test requires a 0.5 ml addition of bacteria cells to 0.5 ml of test solution which results in the 50 percent test solution. Therefore, the highest concentration that can be tested is a 50 percent effluent concentration. Second, the color of effluents poses a problem due to certain colors masking the luminescent output from the bacteria. Therefore, a reduction in light may be due to either the pollutant's effect on the bacteria or due to the color absorbing the light. Third, some tests have been providing false negatives. False negatives are defined as where lethality has occurred in the tests with fish and/or invertebrates, but the Microtox test results have indicated no significant change or an increase in the luminescence from the bacteria. Therefore, if you are using the unit as the sole tool in determining an effect, you're going to be misled in some cases. So far, between 10 and 20 percent of the effluents tested have resulted in false negatives. Fourth, fresh water effluents require that sodium chloride be added to bring the salinity up to 20 parts per thousand prior to testing. The reason for this is the bacteria used in the test is a saltwater species. Therefore, there is some question from the regulatory aspect on the validity of the results obtained from testing an effluent sample to which some compound has been added that may change the effluent chemical characteristics. Representatives from Beckman Instruments are fully aware of all the disadvantages and are working to resolve the problems. They have indicated recently that they may have a breakthrough in the color problem, and may also have a technique for increasing the effluent concentration tested. In Region IV, the Microtox unit will certainly not replace fish toxicity tests in the regulatory requirements; however, once the problems are resolved, it may be used as an additional tool in the aquatic toxicologist's tool box for screening effluents for toxicity. Also, keep in mind that the findings from the evaluators testing pure compounds may be entirely different than what Region IV has found with the complex effluents.

Also, there is one last topic I would like to cover briefly which involves an instrument which was developed and is being used in Europe to monitor toxic effluents. Region IV[1] is presently evaluating this instrument which is called TOXIGUARD and is manufactured by EUR-Control. The company has an office in Decatur, Georgia. The Europeans are using it as an on-line monitor of raw waste before to the wastewater goes into a treatment system. The instrument utilizes bacteria growing on multiple plates with a small continuous flow of wastewater passing through an aeration chamber and then through a chamber containing the bacteria growing on the multiple plates. If the wastewater is not toxic and the bacteria are not stressed, then normal biological activity occurs and the DO in the wastewater is removed. As the wastewater exits the chamber, it passes across a DO probe which monitors the DO level. If there is zero DO, then the bacteria are not stressed, and it is assumed that the wastewater is not toxic. However, in the event the DO begins to increase and it approaches a pre-determined milligrams per liter, an alarm bell goes off, and the operator can divert the wastewater to a back-up lagoon so it won't kill the bacteria in the treatment system. We're starting to evaluate the instrument using complex effluents and comparing the results with the results obtained from acute toxicity tests with fish.

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[1] As of November 1979, Region IV is no longer evaluating the TOXIGUARD.

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BIOCONCENTRATION TESTS FOR EFFLUENTS

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One of the objectives of biomonitoring programs for complex effluents is to select the most cost-effective set of tests which provide reasonable assurance that potential hazards of a discharge have been evaluated. Standards for BOD have been in use for a long time, yet it is obvious that many highly hazardous chemicals could be discharged even though the BOD standards are met.

Although recent studies indicate that most effluents receiving proper secondary treatment show little acute toxicity to aquatic organisms, acute tests are the best experience tests to conduct on an effluent after BOD has been removed. The next level of concern is the chronic toxicity of the waste stream which could be determined from the more costly embryo-larval test. Finally, even if testing showed no hazard from the above tests, chemicals such as PCB, hexachlorobenzene, etc. could be discharged at quantities great enough to cause hazardous residues in fish in the receiving waters. The reason is that these chemicals are extremely bioaccumulative and will be found as concentrated residues even when the concentration in the water is below a toxic level. Consequently, there is a need for a test for bioaccumulable chemicals and a need for a strategy for using the test in an appropriate manner.

First of all, let me just define some terms. The bioconcentration process is the accumulation of chemicals in the body of an animal over and above the ambient concentration. In the case of fish, it's simply the amount in the fish tissue compared to the water environment or the food. There's been an argument in the literature whether fish bioaccumulate more through the gills by respiratory uptake and partitioning into the fish, or through the food chain. The answer is that both mechanisms are important, and the question of how important depends on the environment in a very predictable way. The bioaccumulation process is the sum of bioconcentration and biomagnification; biomagnification is that which is accumulated from ingesting the chemical, and bioconcentration is that which fish can accumulate through the gills when placed in water with that chemical dissolved in it.

The bioconcentration test is conducted by placing approximately 35 fish such as the fathead minnow in a large aquarium. The chemical or effluent is continuously added, and composite samples of five fish are removed after 2, 4, 8, 16, and 32 days for analysis. It is generally observed that the chemical is accumulated in a manner simulated by a first-order process which gives a rapid initial uptake followed by a slower rate and finally a relatively constant residue concentration (Figure 1). This point is called the steady-state concentration for the exposure and the bioconcentration factor is calculated by dividing the steady-state residue by the water concentration.

Reasearch has shown that the ratio between the fish residue and the water concentration of a chemical, i.e., the bioconcentration factor, is constant over a wide range of water concentrations. This means that the steady-state residue in fish can be calculated from the water concentration if the latter is known. Also, the bioconcentration factor for chemicals varies from less than one to greater than 100,000. Chemicals which have small bioconcentration factors generally reach steady-state in fish within the first few days of exposure whereas those with high bioconcentration factors may take several weeks or may never reach a true steady-state condition. Figure 1 shows the uptake of hexachlorobenzene in various age groups of fathead minnows. Although it appears that a steady-state is reached, a closer examination shows that the residue continually increases over the 115-day exposure.

The research we have completed at the Environmental Research Laboratory - Duluth suggests that a 30-day exposure is the longest period of exposure needed to assess the bioaccumulation potential even though the actual accumulation in the environment may be slightly higher for the higher bioaccumulable chemicals. Moreover, the test can be readily adapted for effluent tests using either live cages or a single aquarium receiving the effluent. We have conducted bioconcentration tests concurrently with toxicity tests by analyzing surviving fish from toxicity tests for residues. While the kinds of experiments give a rapid indication of the presence of highly bioaccumulative chemicals, they may not give the same bioconcentration factors obtained from the longer bioconcentration test.

Because bioconcentration tests on complex effluents require a qualitative and quantitative chemical analyses of residues in the exposed fish, the cost of the tests in a monitoring program is still rather substantial. Consequently, we have developed a screening test which is based on the use of bioconcentration factors but reduces the overall costs. We tested chemicals which were representative of a wide range of chemicals and found that the bioconcentration factor could be related to the n-octanol/water partition coefficient as shown in Figure 2. The octanol/water partition coefficient is a measure of the fat solubility of chemicals and is generally inversely related to water solubility. Chemicals such as detergent builders, solvents, phenolics, etc. have a high water solubility and a corresponding low bioconcentration factor. Chemicals such as the chlorinated pesticides, PCB's, and brominated flame retardants have a very low water solubility and a corresponding high bioconcentration factor.

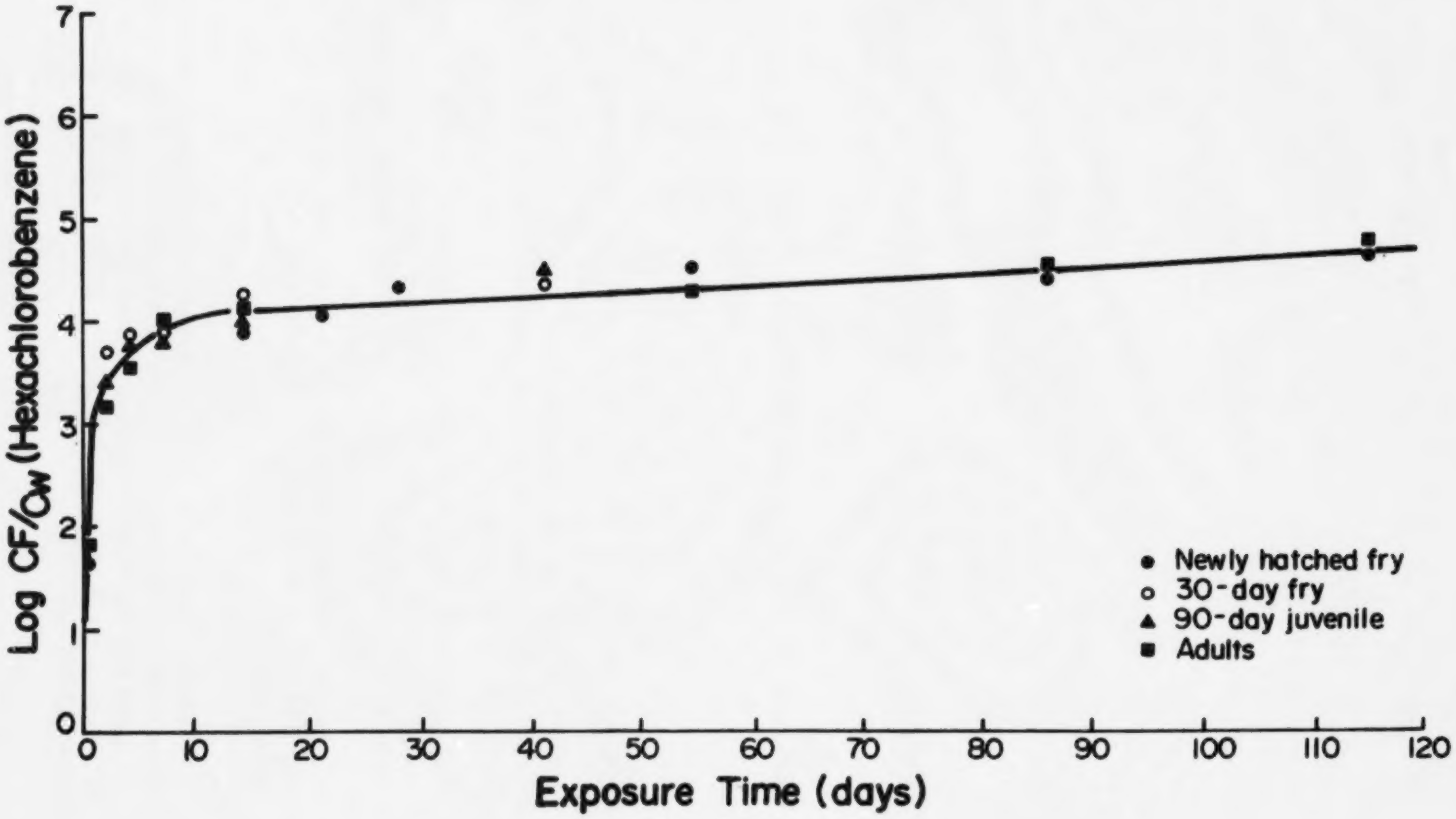

Figure 1. Accumulation of hexachlorobenzene by fathead minnows of four age groups.

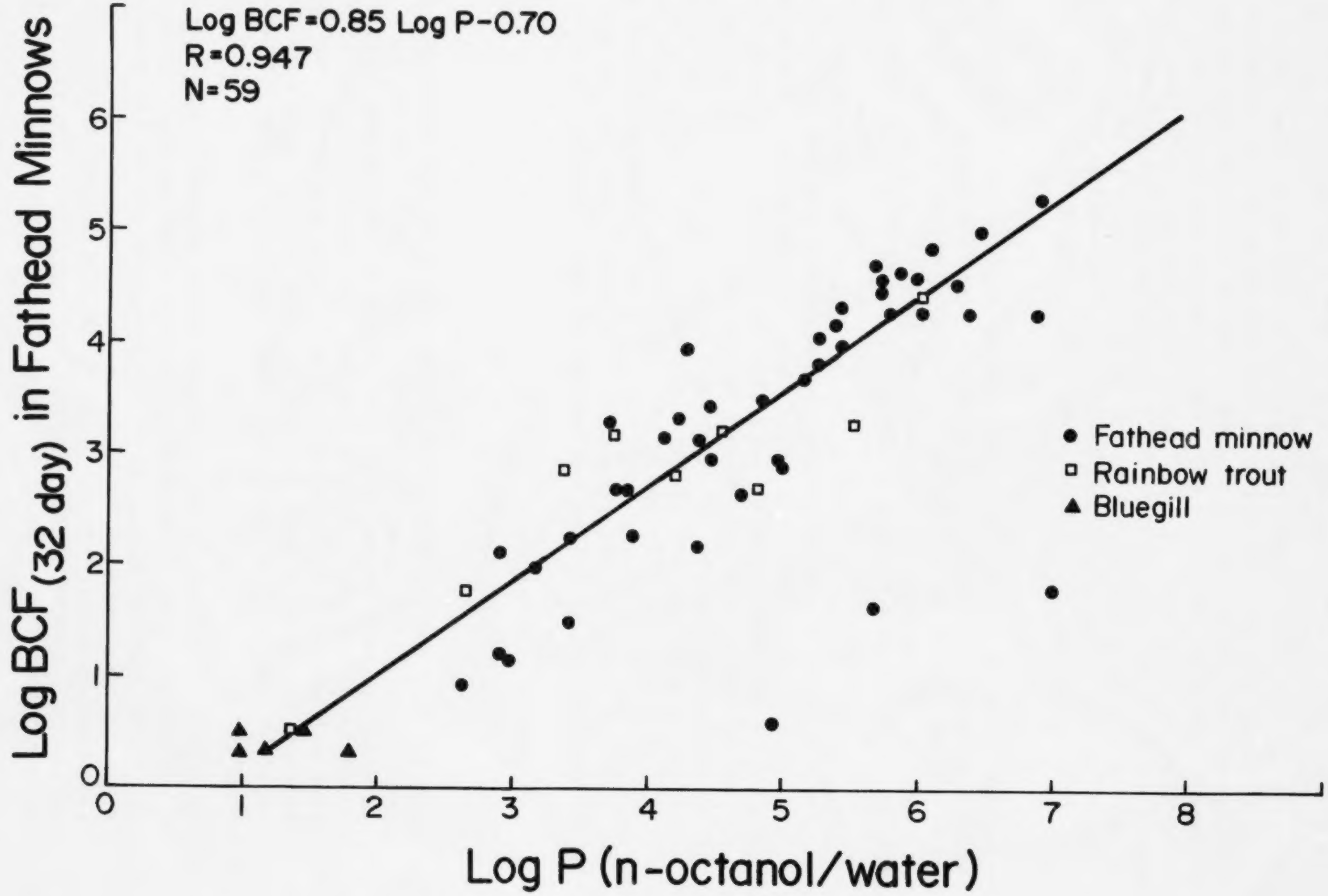

Figure 2. Relationship between the bioconcentration factor of 54 organic chemicals in fish to the log P of the chemical.

This correlation is useful because it will permit estimates of bioconcentration factors for chemicals without testing. Only if those chemicals which have a large partition coefficient are being used in a plant would a detailed bioconcentration test be justified to determine if such chemicals are leaking out of the plant. Since the log P can be estimated from structure, the overall estimate of bioaccumulation potential can be achieved at the screening level at low cost. We have found that chemicals with partition coefficients less than 1000 are unimportant from a bioaccumulation viewpoint, and bioconcentration tests should be limited to those chemicals most likely to be bioaccumulative. Since the majority of industrial chemicals belong to this group, these screening relationships may substantially reduce the cost of testing.

If the effluent or wastewater is so complex that it is impossible to list the possible chemicals, it is still possible to screen the effluent for bioaccumulative chemicals to determine if the bioconcentration test is justified. In the EPA report EPA-600/3-78-049 (available from NTIS), we showed that the partition coefficient of chemicals can be determined from the retention time of the chemical on a reverse-phase liquid chromatography column. Consequently, the retention time of the "peaks" on this column can be used to estimate log P and therefore the bioconcentration factor of chemicals, even though the identity of the "peaks" is not known. Screening for chemicals of high bioaccumulation potential is translated to screening for chemicals which have a long retention time on the reverse-phase column. Since water is used as a solvent, effluent samples can be injected directly onto the column or a precolumn and effluent screened within twenty minutes. If "peaks", or chemicals, are detected which have a long retention time, it is highly probable they will have a large log P and a large bioconcentration factor. If none are detected, it is unlikely a bioconcentration test will provide useful information concerning the hazards of the effluent.

DISCUSSION

Statement by Glen Pratt, EPA Chicago: Let me just clarify one question that was asked earlier today on natural toxicants in an industry's intake water. If you have a material in your intake water and if you do not add any contaminants to it in your process, you are not responsible for it in your effluent. The exception is that if you take water from one waterway or from a well and put it into another waterway, you would be liable if it were of a lower quality than the receiving waterway.

Question: In the last session a question was asked regarding what other jurisdictions were doing with respect to biomonitoring. I might point out that in Canada there is a requirement in the regulations to have industry do bioassay tests, basically 6-hour LC_{50} flow-through tests. Also, to carry on with a question that was raised this morning which addressed the increase in the amount of biomonitoring that's going to be requested by the U.S. from the state agency, I've got a problem with lab availability now, and it may be a problem in the future. Also what kind of quality control problems will there be as a result of the increased workload?

Answer: Are you asking will laboratories be available?

Question: The problem is that now in Illinois I don't know of more than two or three sources that are available commercially to do biomonitoring. For example, if 30 industries have biomonitoring requirements within their permit, how are they going to get the monitoring accomplished? We will get to the point where labs start to spring up, and then what kind of quality assurance are we going to get from these labs?

Answer: I don't have too much concern with the first problem of lab availability so long as the regulatory agencies don't impose unreasonable deadlines in doing this. I think you'll be surprised to find how many labs really are now able to do these tests, and I can assure you that, if the need is there, those who are willing to do them for a fee will also appear. This is state-of-the-art technology rather than research tools at this stage. As far as quality control is concerned, there are a number of round-robin tests being performed right now to measure the precision of these various tests. There are activities by the Office of Toxic Substances and probably state agencies, too, to develop good laboratory practice manuals and offer laboratory certification. So I think there's a lot of activity in the area of quality control.

Question: We're looking at a lot of bioassay tests today, and I'm wondering what criteria is used in determining which test might be suitable for any given waste stream?

Answer: It's my view that this should be based on an industry-by-industry, plant-by-plant basis. What we're trying to do today is to give you the broad range of tests that are available. In our work and the state's work with industries, we need to look back at the specific processes in the plant. This is the one reason why we believe it's tied with process evaluation. Are you looking at a type of compound in the plant that you think would tend to be a long-term bioaccumulative material?

Are you looking at a process in a plant that produces only an intermediate compound which is consumed within the plant? When Don Mount says you can't generalize, we totally agree, and this is why we're not having a broad spectrum requirement that every industry must do test A, B, or 2 from Column A and one from Column B. Instead, we're saying that it should be a plant-by-plant evaluation. If you are in an industry that doesn't have biologists on its staff, you might want to get together with either the state agency or a biological consulting firm to see which particular test would be suitable for your given facility.

Answer #2: In the way of clarification, in case it slipped by some of you, the bioconcentration tests that Gil described is directed at a phenomenon or an effect that we're concerned about at levels below those concentrations causing toxicity. So if you have a direct toxicity problem, you may not be concerned with the bioaccumulation test at this point. I think it is clear from everyone I've talked to that it is not prudent to require all of these tests by any means, but rather to select those that are most appropriate to the problem that you have.

SEDIMENT BIOASSAY

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Max Anderson

The need for a sediment bioassay in Region V became quite apparent in 1976 and 1977 when we encountered an increase in dredging activities in the harbors and streams around the Great Lakes. At that time, Rock Prater was working for the Central Regional Laboratory on a special assignment. Through a combined effort, we adapted an apparatus that was originally described by Cal Fremling of Wannona State College and have been using that as our sediment bioassay chamber. The chamber is depicted in Figure 1.

The chamber is constructed entirely of glass and is a closed cycle system. An aerator forces air and water to circulate throughout the system. The sediment is deposited in the upper part along with the organisms that are being tested. The small cup on the top left side is used for the Daphnia bioassay. We place other organisms within the chamber itself. If we're using fish as our test organisms, they are placed in one of the larger glass jars. The cost of this particular apparatus runs between $30 and $50, so it's not very expensive and it works quite well.

We have adapted and enlarged this apparatus at the Central Regional Lab for use in long-term biomagnification work that is just getting started. Since we're only using fish in this particular study we find that we can maintain them for a longer period in the larger unit. The principle is exactly the same as the smaller unit but it is constructed of a 10 gallon aquarium on the top and two 5-gallon aquariums underneath (Figure 2). In Figure 3 we have two tandem units together. Figure 4 shows a bank of units in our environmental chamber. We estimate that the cost for setting up

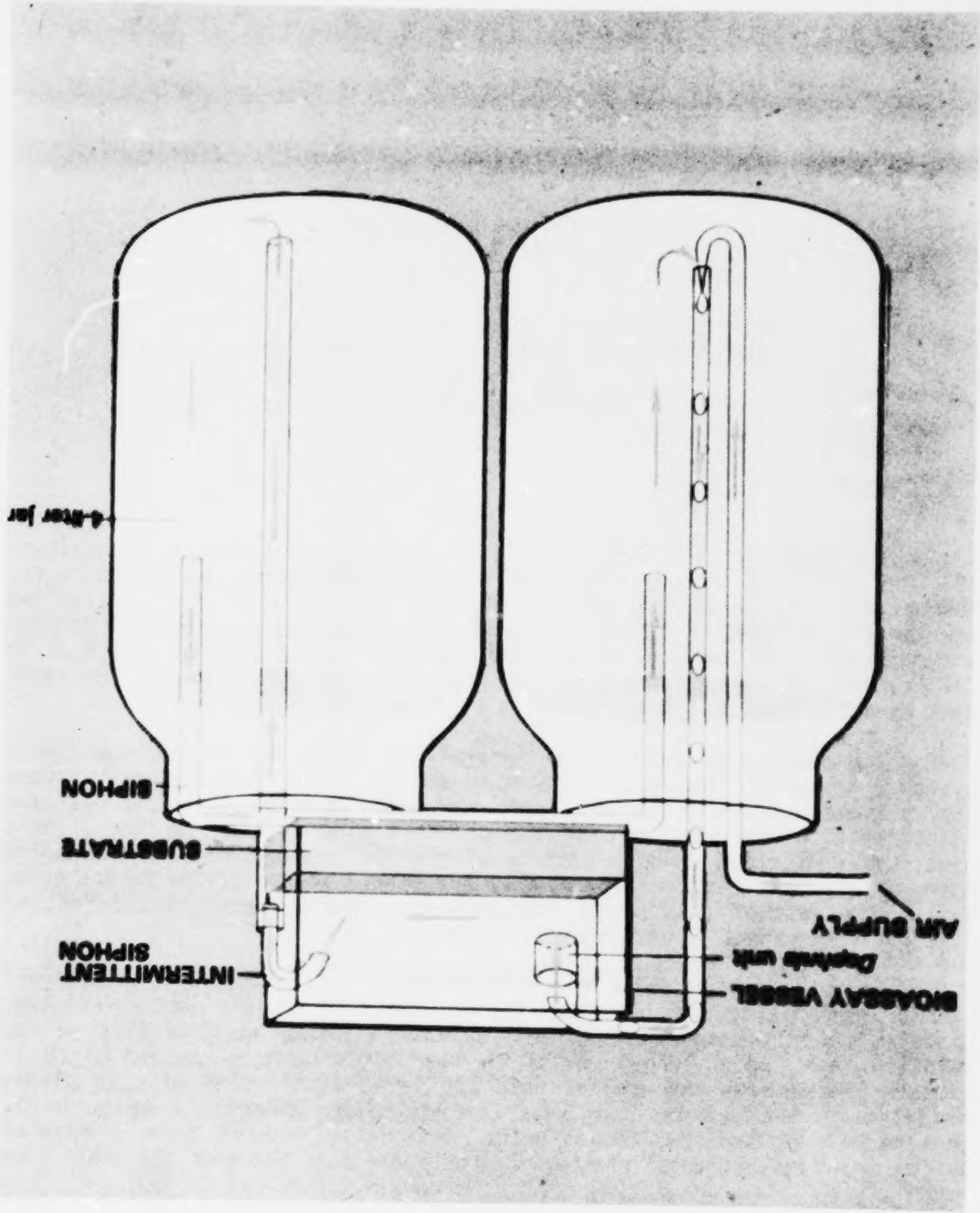
4-liter jar
SIPHON
SUBSTRATE
INTERMITTENT SIPHON
AIR SUPPLY
Daphnia unit
BIOASSAY VESSEL

Figure 1. Schematic diagram of a closed-cycle sediment bioassay chamber.

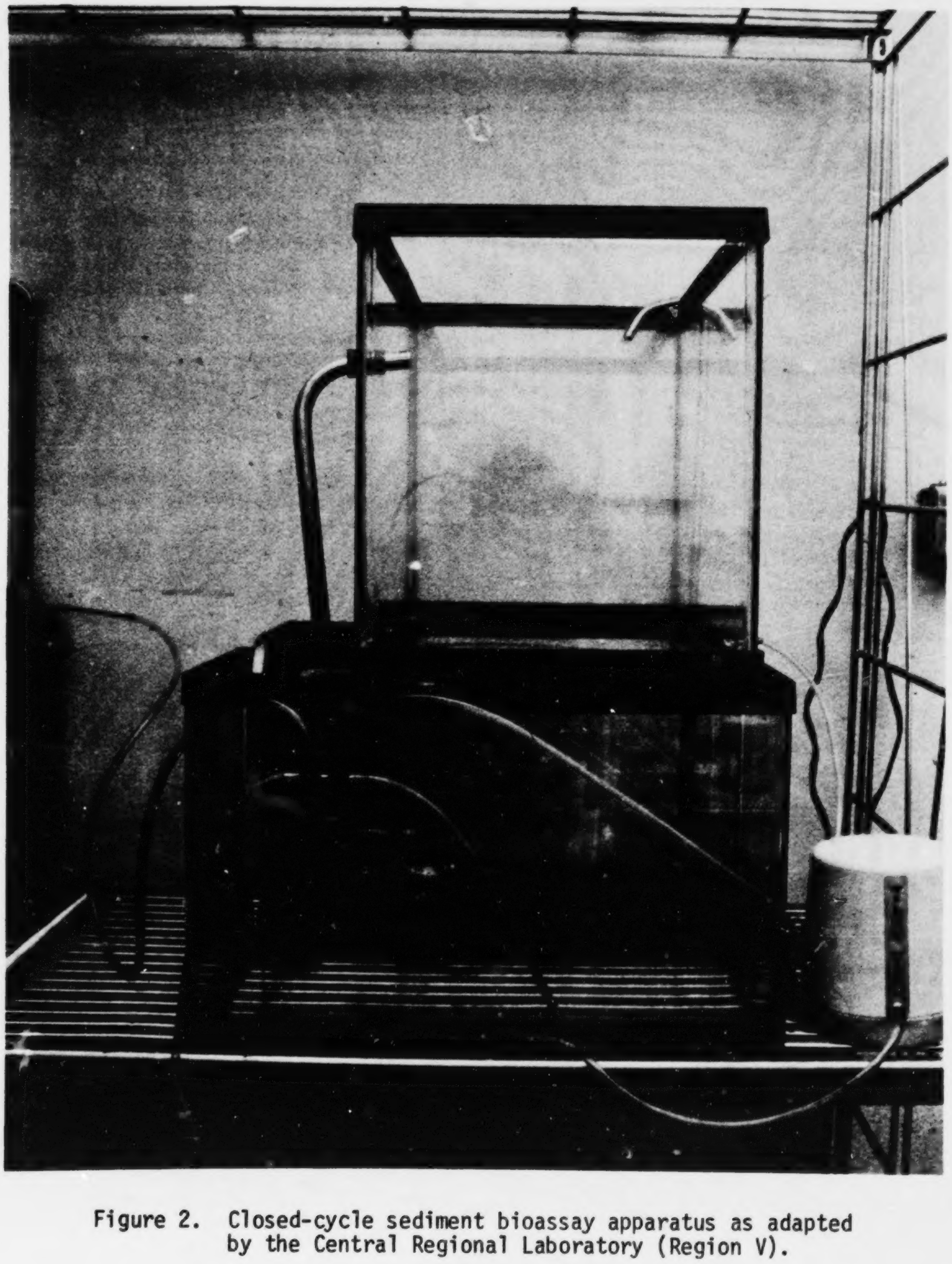

Figure 2. Closed-cycle sediment bioassay apparatus as adapted by the Central Regional Laboratory (Region V).

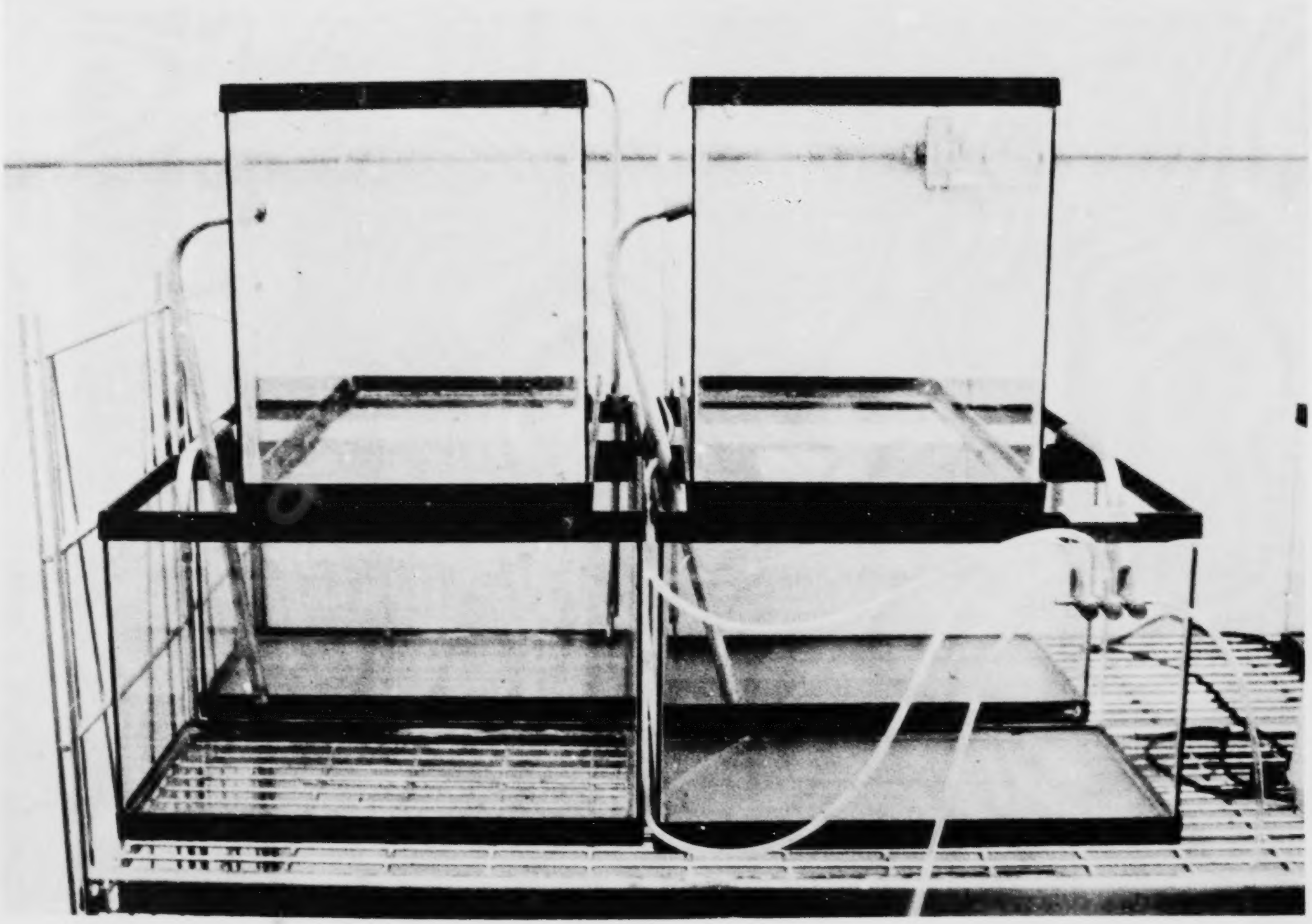

Figure 3. Two tandem closed-cycle bioassay units (Central Regional Laboratory, Region V).

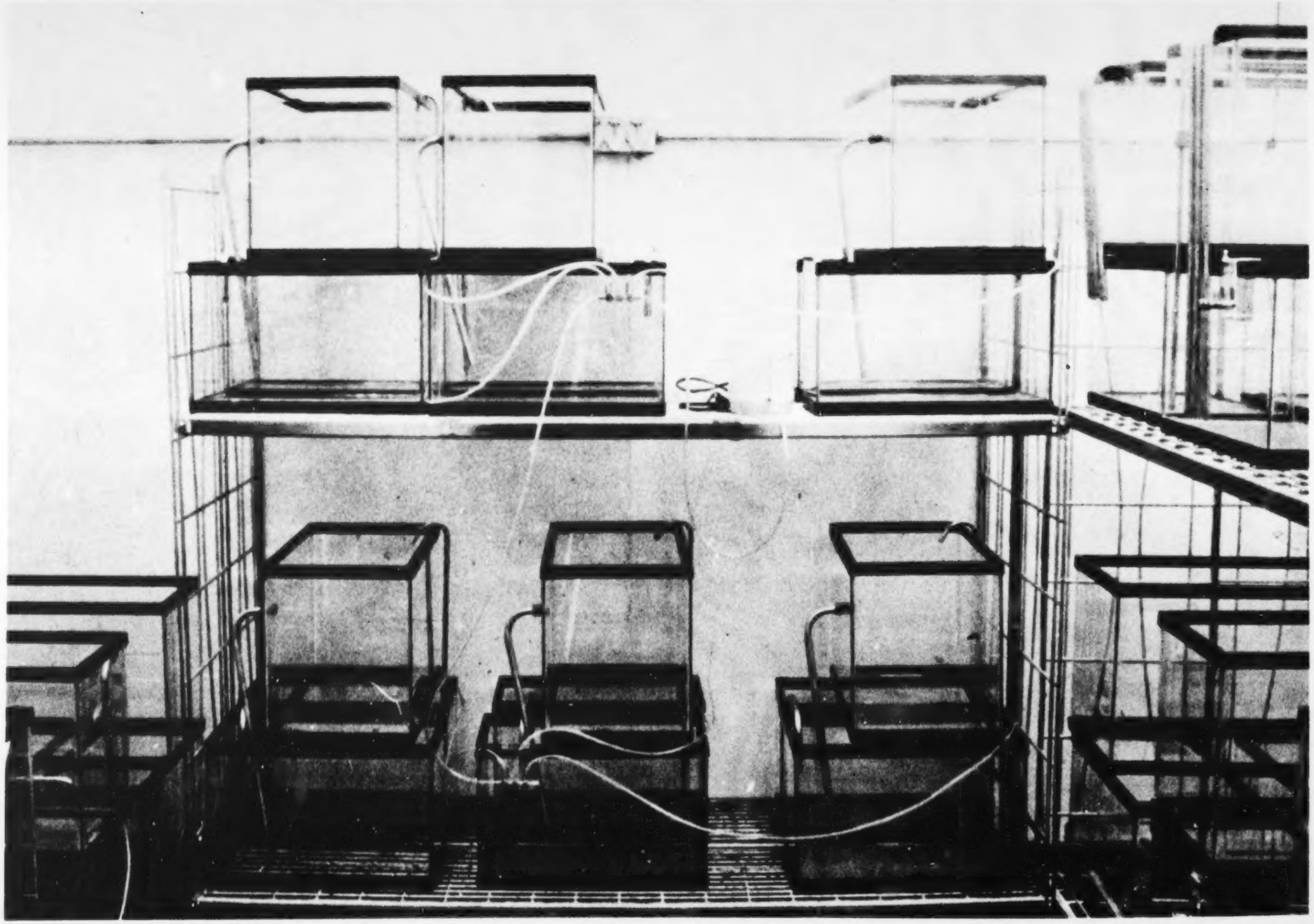

Figure 4. Bank of closed-cycle sediment bioassay units (Central Regional Laboratory, Region V).

one of these unit would be about $75 to $100, which is relatively inexpensive if you're interested in a long-term biomagnification study.

I would like now to turn the time over to Rock Prater who is going to discuss the application of this sediment bioassay apparatus based upon some extensive work that he has done.

Rock Prater

It never fails to amaze me that, after 21 years of teaching, I still get nervous standing in front of a group. Not to use this platform as a business opportunity, but as of last Friday, I am no longer with Heidelberg College; I'm with a consulting firm that's doing primarily sediment bioassay work.

As Donald Mount said in his introduction this morning, there are two accepted approaches to determining the condition of an aquatic ecosystem - animal counts in standing crop and identification of chemical parameters. Although the ecosystem might contain an acceptable set of chemical parameters, there are situations in which no animals are present. Unfortunately, the impact of the sediments in such a situation is usually overlooked.

This particular apparatus that we are currently using for sediment bioassay is quite basic; however, I think that the data that we have accumulated after investigating harbors on all the major lakes, with the exception of Lake Ontario, shows that it seems to be working quite well. The apparatus still needs refinement; we are soliciting a grant to continue development. Mr. Robert Hoke, who is also with Aqua Tech, will be a valuable asset since he has just finished three years of graduate research in this area.

We have used this apparatus in both riverine systems and lakes. Figure 1 shows a small creek which receives effluents from four major industries. One of these, the "oil refinery", was being taken to task by EPA for thermal pollution of this stream. We took samples above and below the major outfalls to determine what the bottom type was like.

We used three organisms in this bioassay - *Hexagenia limbata*, *Asellus communis* and *Daphnia*. *Hexagenia limbata* is a burrowing mayfly which is especially useful if you're going to look at the effects of sediment. *Asellus communis* is an isopod that lives at the interface, and *Daphnia* is a water flea which lives within the water column. In other studies we usually use fish.

Figure 2 indicates the toxicity to the three major species that we used. Proceeding downstream from the control station we get 100 percent mortality during the 96 hours, a small amount of recovery, and then an increase. But what does this mean in relationship to the refinery? Should they be forced to eliminate the thermal discharge when indeed the sediment types that currently exist (and we did profiles on these) might prevent the existence of a healthy ecosystem? In addition, the seiche effect on this system would prevent any scouring so that the return of a healthy ecosystem may take a number of years or in fact may never occur. This is a typical example of the need for a sediment bioassay. If we simply look at the water column, or at the standing crop of organisms, and ignore the sediment type we may be amiss.

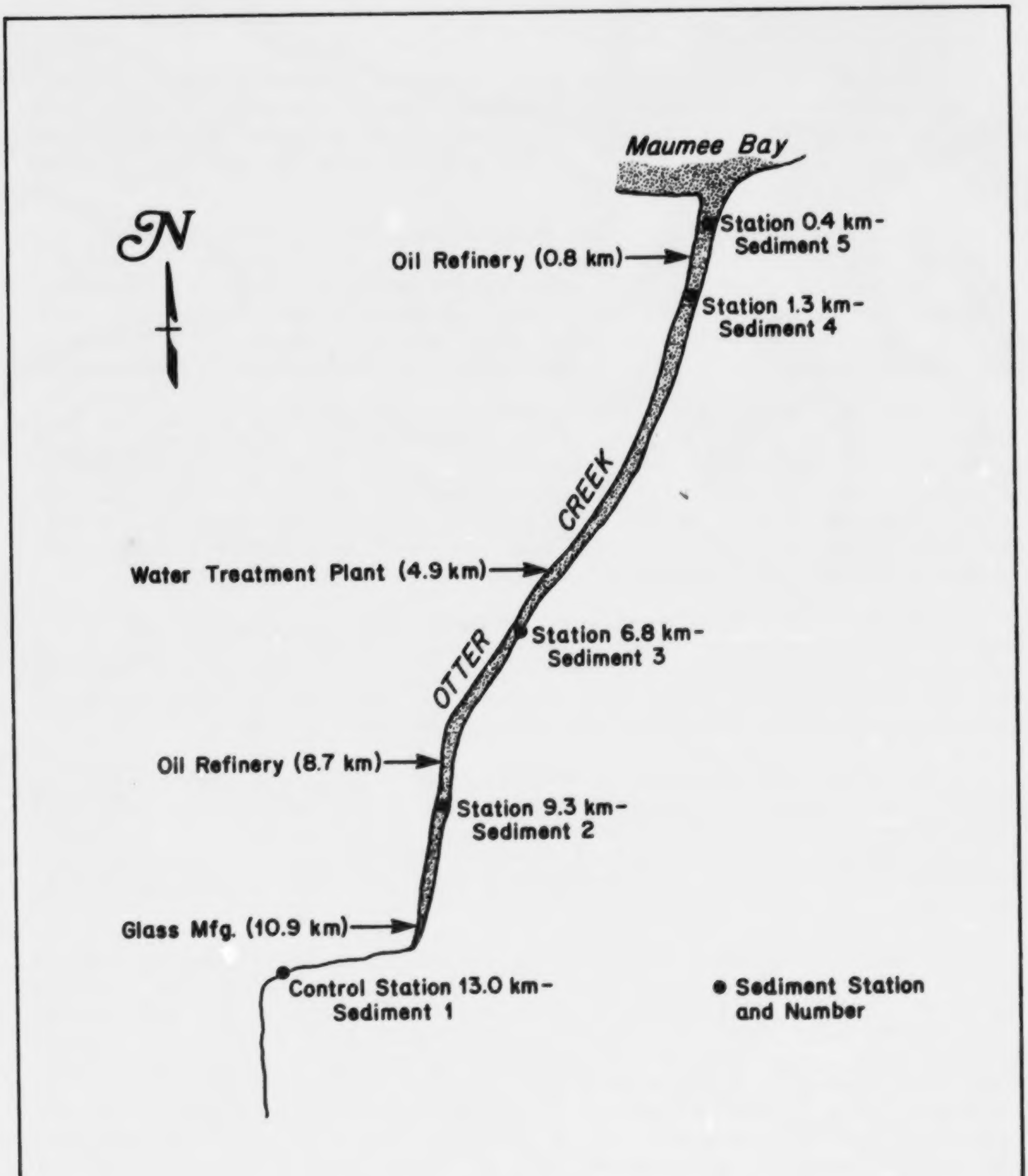
Maumee Bay
N
Station 0.4 km-
Sediment 5
Oil Refinery (0.8 km)
Station 1.3 km-
Sediment 4
OTTER
CREEK
Water Treatment Plant (4.9 km)
Station 6.8 km-
Sediment 3
Oil Refinery (8.7 km)
Station 9.3 km-
Sediment 2
Glass Mfg. (10.9 km)
Control Station 13.0 km-
Sediment 1
● Sediment Station
and Number

Figure 1. Sediment stations and industry locations on Otter Creek, Ohio (not to scale).

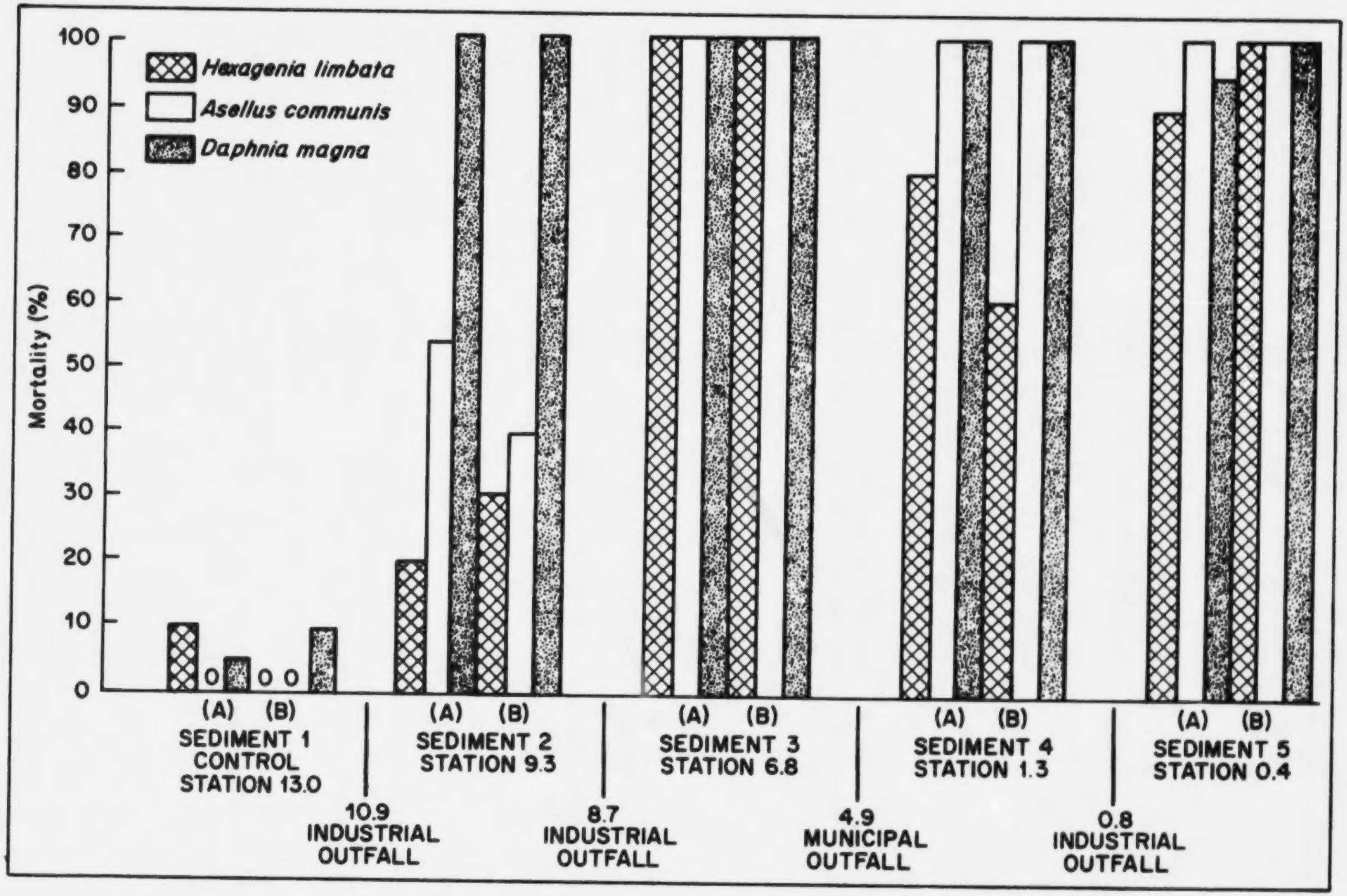

Figure 2. Percent mortality of Hexagenia limbata, Asellus communis, and Daphnia magna during a 96-hour sediment bioassay of Otter Creek, Ohio, 1976. Replicates are indicated as (A) and (B). Stations are numbered in kilometers from the mouth of Otter Creek (not to scale).

This technique can also be used in making management decisions. We are currently under contract with the Corps and private industry to study a number of harbors. The situation that exists in Indiana Harbor is a good example since it is generally recognized that Indiana Harbor is a toxic environment.

If the harbor is dredged, how do we dispose of the dredged material? Should we dump it into the open lake, or should we take it upland? The sediment bioassay can be used to help answer this question.

In Figures 3 and 4, it is evident that the toxicity of the sediments decreases as we go from the harbor to the open lake. Since the toxicity of the dredged material varies between locations, the disposal technique should vary accordingly. If the alternative is to dispose of the toxic material upland, it still must be capped. The most reasonable approach, then, would then be to use the less toxic material as the cap, since it would have to be dredged anyway.

Another example is Ashtabula Harbor. We did a study of the upper layers only. Using this sediment bioassay apparatus, we determined that these upper layers were only moderately polluted or nonpolluted. The results led me to conclude that the dredged material should be allowed to be disposed of in the open lake, which is what I would normally recommend for moderately polluted or nonpolluted sediments. However, EPA did not agree. They wanted to know if the toxicity changed with different depths of the sludge.

So, in the next series of samples we looked at the toxicity at different levels. According to the results presented in Figure 5, the top layer of site 6 (Ash-6T) was more toxic than the bottom layer (Ash-6B). However, at site 2, the top layer was less toxic than the bottom layer. If you must decide how deep to dredge and where to put the dredged material, this type of information is a necessity. If the top 6 feet are not very toxic but the underlying sediments are, it makes sense to dredge to the six foot level, dispose of the nontoxic material in the open lake and do something else with the underlying material.

Unfortunately, Figure 5 indicates that the replicability is not as good as we would like. But anyone who has done chemical analysis on sediments will admit that replicates can vary considerably.

Basically, that is the sediment bioassay program that we are using to determine what we are going to do with dredged spoils from harbors and ship slips in the Great Lakes. It is my opinion that there will be more sediment bioassay work down the road. Although it needs refining, it is a very substantial technique.

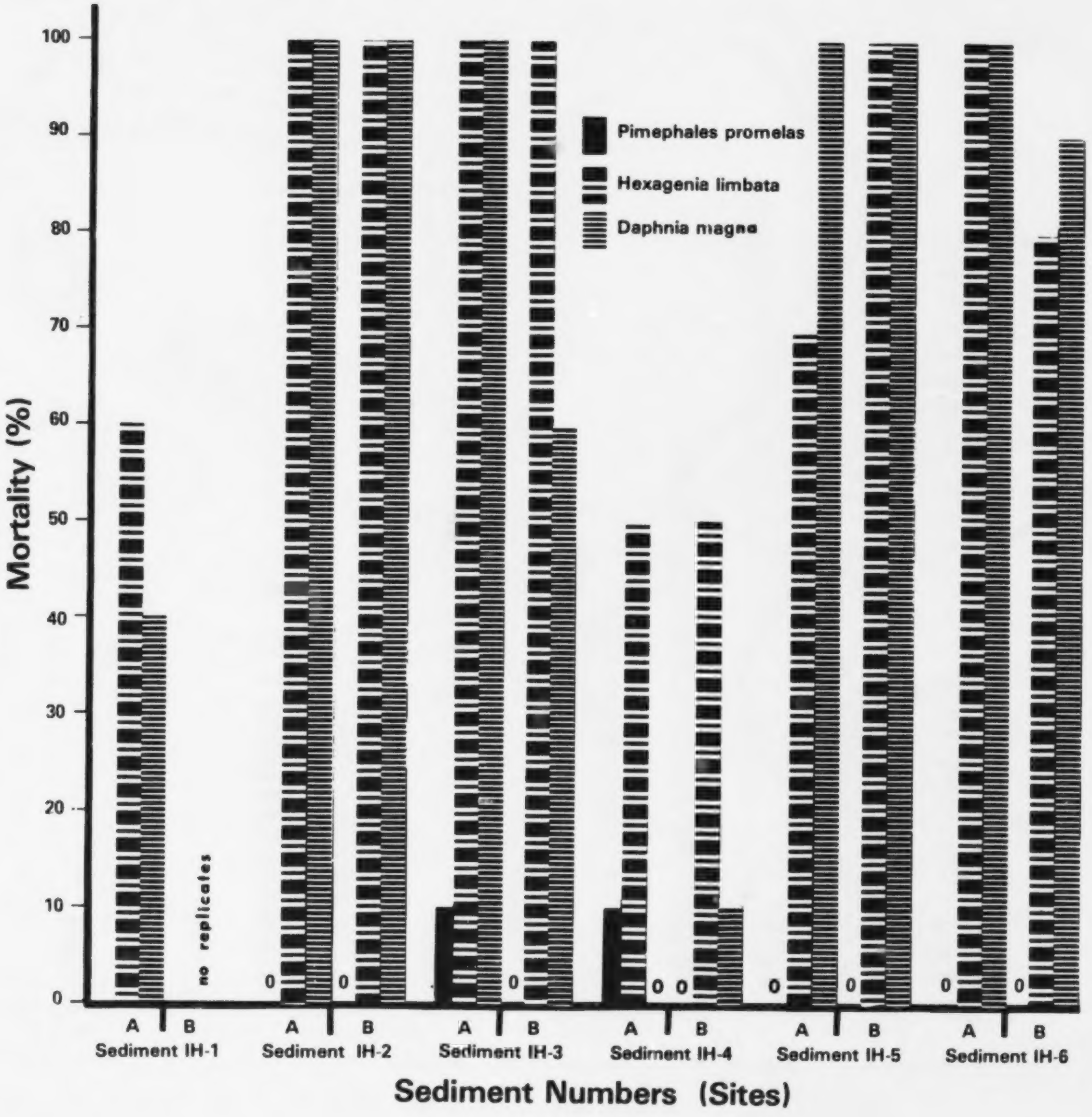

Figure 3. Percent mortality of Hexagenia limbata, Daphnia magna, and Pimephales promelas during a 96-hour sediment bioassay of Indiana Harbor, Indiana, 1978.

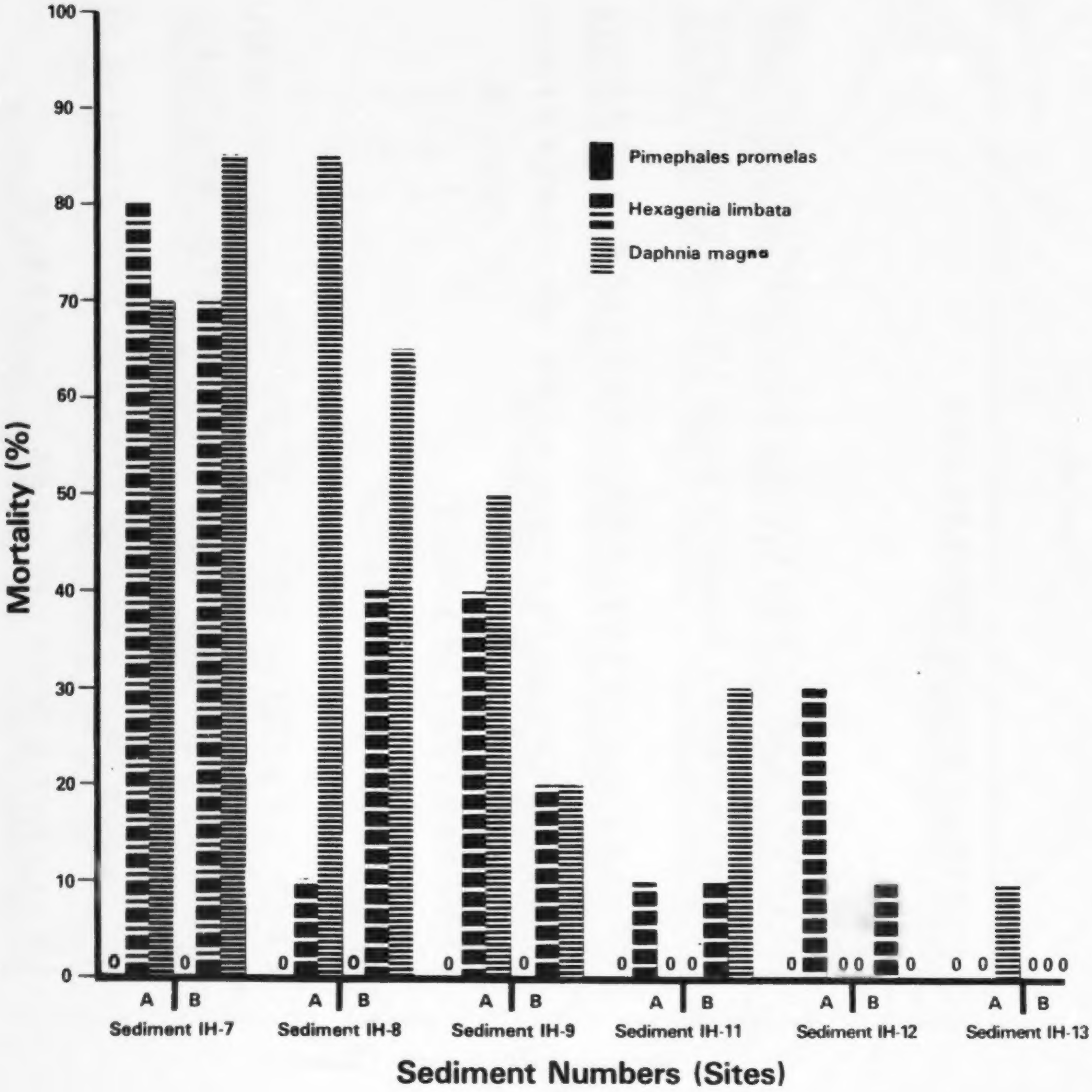

Figure 4. Percent mortality of Hexagenia limbata, Daphnia magna, and Pimephales promelas during a 96-hour sediment bioassay of Indiana Harbor, Indiana, 1978.

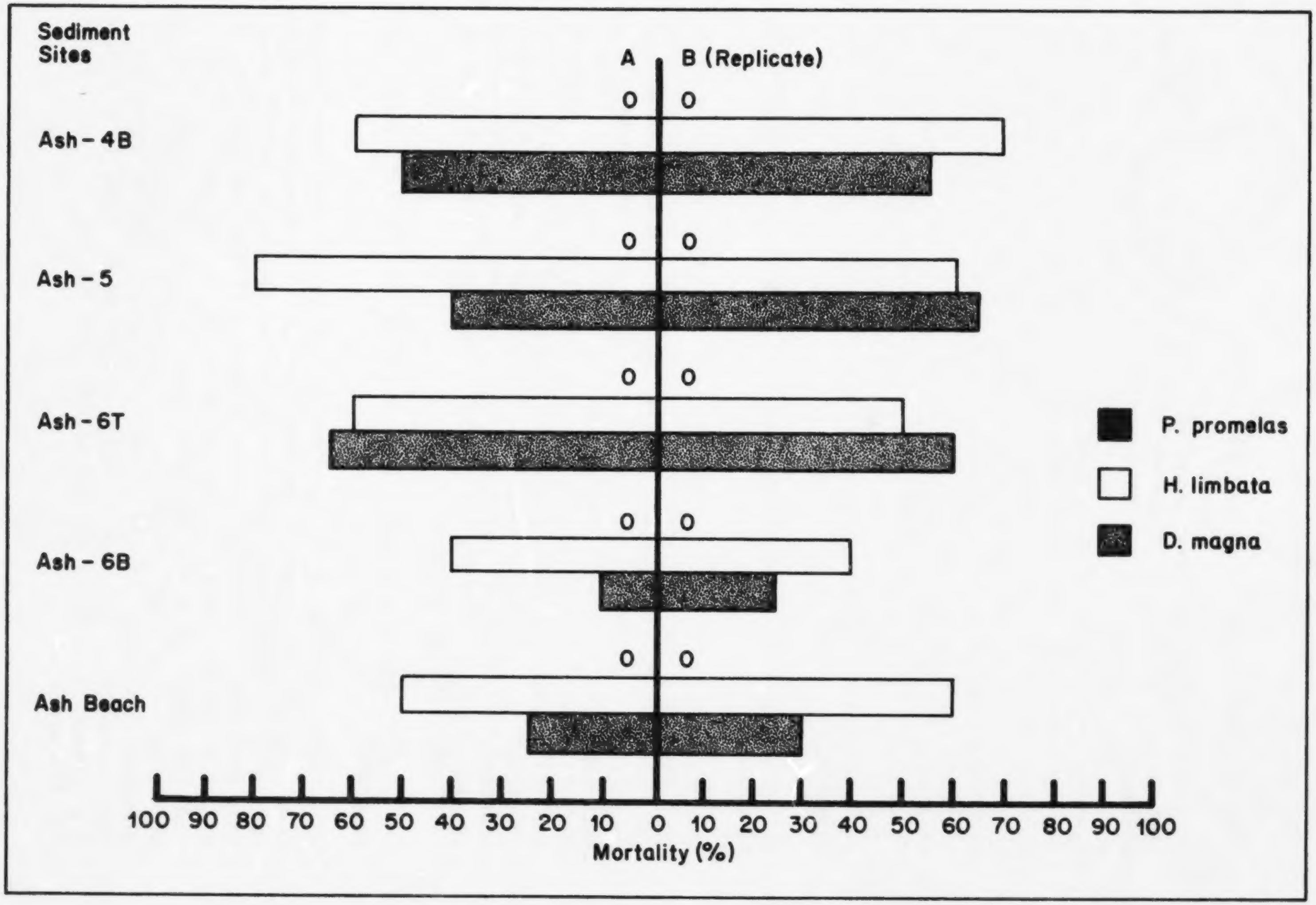

Figure 5. Percent mortality of *Pimephales promelas*, *Hexagenia limbata*, and *Daphnia magna* from a 96-hour sediment bioassay of Ashtabula Harbor illustrating different toxicity for surface samples (T) and bottom sediments (B).

DISCUSSION

Question: What is the time of the exposure?

Answer: 96 hours, basically. What we tried to do was develop a procedure that you can do in a short period of time because there are about 126 harbors in Region V that are proposed to be dredged. It's not practical to run six to eight week tests on 100 or more harbors. So what we need is some sort of test that's a red flag. I'm not proposing that this be used in lieu of bulk analysis or chemical analysis and bioassay of the elutriate. I'm saying it should be used in conjunction with them. We've just put the old elutriate chemistry and bioassay argument to rest. I believe Bob has been working on a lengthy statistical analysis using a large data base to see when elutriate chemistry and bulk chemistry is important and how they relate to percent mortality. Why 96 hours? That just seems to be accepted exposure. Generally speaking, if you've got very toxic sediment, you'd get the same results in 24.

Question: If you're looking at sediment, why do you have the flow-through apparatus?

Answer: You get a high BOD with sediments. If dissolved oxygen were to drop down to 1 part per million, let's say, and the critters die, you can't say it's because of the sediment toxicity. So the recycling is important in maintaining dissolved oxygen within a reasonable level. Very seldom does it drop down below 5 parts per million. Plus, if you're in a riverine system, we can almost duplicate the flow because you can change the calibration on the apparatus and actually have a flow-through system that might approach the CFS's; or you can change the flow across the pattern in the sediment. Primarily, though, we use continuous flow to maintain dissolved oxygen.

Question: Are your critters available from stock or do you cultivate them yourself?

Answer: The last study we did, we got the *Daphnia* from the Toledo Zoo. *Daphnia magna* we had Dr. Kreiger do; they are quite readily available. *Pimephales*, the cosmopolitan that was talked about this morning, is readily available here. As for the quality, we get ours in Newtown, as long as we're doing governmental work. The *Hexagenia* are called wigglers, and we get them from a bait shop at $7 per thousand. The proprietor thinks I'm the greatest fisherman in the world, I guess. *Asellus communis* - that's another story. We maintain a culture unit and keep these. The biggest hassle is in the winter; you've got to be prepared for the winter.

Question: About the relationship between toxicity with *Daphnia* and toxicity with your other test animals - it appears as if the *Daphnia* was more sensitive most of the time. Is there any advantage to using an additional three species or should you just go with that?

Answer: Section 404 says that Thou Shalt use three. So you must use multiple species, number one. And I think that we got the spectrum. *Daphnia* is very sensitive, and *Pimephales* I think you'd have to hit with a sledge hammer. I think the other two species fall somewhere in between. *Daphnia* is very erratic; it is very tough. Sometimes we use clean sand controls and get a 30% kill with *Daphnia*. Maybe they're eating the sand grains, I don't know.

Question: Does your sedimentation take into consideration acclimation?

Answer: Yes it does.

Question: How long do you acclimate?

Answer: Generally, 24 hours. We've found that we get a recolonization in the sediment very similar to the recolonization that existed in the sediment when we took it. In other words, we've looked at the different horizons after we put it in and let it settle out.

TENTATIVE GUIDELINES FOR
FLOW-THROUGH EARLY LIFE STAGE TOXICITY TESTS WITH FATHEAD MINNOWS
FOR USE IN THE U.S. EPA, OTS-ORD ROUND ROBIN TEST

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The following draft protocol for an embryo-larval test is being evaluated for adoption. Users should bear in mind that changes will be made before finalization.

Tests as described in this method estimate chronic toxicities as measured in life cycle tests in a high percentage of the comparisons that currently can be made with the existing data base.

1. In an Early Life Stage Toxicity Test with fathead minnows, organisms are exposed to toxicant during part of the embryonic stage, all of the larval stage, and part of the juvenile stage. The organisms are examined for statistically significant reductions in percent hatch, percent survival, and weight in order to determine upper and lower chronic values.

   A lower chronic value is the highest tested concentration (a) in an acceptable chronic test, (b) which did not cause the occurrence (which was statistically significantly different from the control at the 95 percent level) of any specified adverse effect, and (c) below which no tested concentration caused such an occurrence.

   An upper chronic value is the lowest tested concentration (a) in an acceptable chronic test, (b) which caused the occurrence (which was statistically significantly different from the control at the 95 percent level) of any specified adverse effect and (c) above which all tested concentrations caused such an occurrence.

2. Not enough information is currently available concerning early life stage tests with fathead minnows to allow precise specification

of details for most aspects of the test. Enough such tests have been conducted and enough aspects have been studied, however, to indicate that these guidelines are appropriate. A prudent course of action for anyone planning to conduct such tests would be to initially conduct a test with no toxicant to gain experience and to determine if the requirements of items 10, 11, 19, 20, 25 and 26 are met using the planned water, food, procedures, etc. General information on such things as apparatus, dilution water, toxicant, randomization of test chambers and organisms, and methods for chemical analyses, can be found in Draft #10 of the proposed ASTM *Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*.

3. Tests should be conducted with at least five toxicant concentrations in a geometric series and at least one control treatment. The concentration of toxicant in each treatment, except for highest concentration and the control treatment, should usually be 50 percent of that in the next higher one.

4. If a solvent other than water is used to prepare test solutions, a solvent control (at the highest solvent concentration present in any other treatment) is required in addition to the regular control, unless such a control has already been tested in the same water with the same species of fish, food, and test procedure and the water quality has not changed significantly. A concentration of solvent is acceptable only if it is (or has been) shown that that concentration or a higher one does not cause a difference (increase or decrease in any of the kinds of data specified in item 27) from control organisms that is significant at the 95 percent level using a two-tailed t-test.

5. For each treatment (toxicant concentration and control) there must be at least two replicate test chambers each containing one or more embryo cups with at least 50 embryos divided equally between the embryo cups at the beginning of the test.

6. Two test chambers have been used routinely:

   a. Twenty fish have been tested in a chamber which is 16 cm x 44 cm x 18 cm high with a 16 cm x 18 cm, 40 mesh stainless steel screen 6 cm from one end, with a water depth of 12.8 cm and with a flow rate of 190 ml/min.

   b. Fifteen fish have been tested in a chamber which is 6.5 cm x 17.5 cm x 9.5 cm high with a 6.5 cm x 9.5 cm, 40 mesh stainless steel screen 2 cm from one end, with a water depth of 4.4 cm and with a flow rate of 15 ml/min.

All of the above are inside dimensions. In both test chambers the water depth is controlled by a standpipe located in the smaller screened compartment with the test solution entering at the other end of the test chamber.

7. Embryo cups should be glass cylinders about 4.5 cm inside diameter and about 7 cm high with 40 mesh nylon or stainless steel screen glued to the bottom. The embryo cups must be suspended in the test chamber is such a way as to ensure that the organisms are always submerged and that test solution regularly flows into and out of the cup without agitating the organisms too vigorously. A rocker arm apparatus driven by a 2 rpm motor and having a vertical travel distance of 2.5 to 4.0 cm has been successfully used, as have self-starting siphons that cause the level of solution in the test chamber to rise and fall.

8. An acceptable dilution water for early life stage toxicity tests with fathead minnows is one in which the species will survive, grow, and reproduce satisfactorily.

9. A 16-hour light and 8-hour dark photoperiod should be provided. A 15- to 30-minute transition period at "lights on" and "lights off" may be desirable. Light intensities from 10 to 100 lumens at the water surface have been used successfully, but the intensity should be about the same for all test chambers. Light should be provided by wide-spectrum (color rendering index > 90) fluorescent lamps.

10. Tests should be conducted at 25°C. The temperature in each test chamber should be between 24 and 26°C at all times and must be between 20 and 28°C at all times. If the water is heated, precautions should be taken to assure the supersaturation of dissolved gases is avoided and total dissolved gases should be measured at least once during the test in the water entering the control treatment.

11. The dissolved oxygen concentration should be between 75 and 100 percent saturation at all times in all test chambers. At no time during the test should one test chamber have a dissolved oxygen concentration that is more than 1.1 times the dissolved oxygen concentration occurring in another tank at the same time.

12. The flow rate of test solution through the test chambers must be great enough to maintain the dissolved oxygen concentration (see items 11 and 22) and to ensure that the toxicant concentrations are not decreased significantly due to uptake by test organisms and material on the sides and bottoms of the chambers.

13. A test begins when embryos in embryo cups are placed in test solution and ends 32 days later.

14. Embryos and fish should not be treated to cure or prevent disease or fungus before or during a test.

15. Embryos should be obtained from a fathead minnow stock culture maintained at 25°C and a dissolved oxygen concentration between 75 and 100 percent saturation with a 16-hour light and 8-hour dark photoperiod. Frozen adult brine shrimp have been successfully used as

a food for adult fathead minnows. The most eggs have been obtained in a 30 cm x 60 cm x 30 cm deep chamber with a water depth of 15 cm when 15 cm x 30 cm quadrants are formed with stainless steel screen and one male, one female and one or two substrates are placed in each quadrant. Half-round spawning substrates with an inside diameter of 7.5 cm and a length of 7.5 cm have been used successfully.

16. The afternoon before a test is to begin, all of the substrates should be removed from an appropriate number of tanks in the stock culture unit and should be replaced about the time the lights are turned on the next morning. Enough (at least three) substrates with embryos on them should be removed six hours later and soaked in dilution water for two hours. For each individual substrate the embryos should be gently separated and removed and visually examined using a dissecting scope or a magnifying viewer. Empty shells and undeveloped and opaque embryos should be discarded. If less than 50 percent of the embryos from a substrate appear to be healthy and fertile, all the embryos from that substrate should be discarded. Single embryos with no fungus or partial shells attached are preferable, although embryos with some fungus or partial shells attached and clumps of two or three embryos (with or without separation) have been used successfully. An approximately equal number of acceptable embryos from one substrate should be impartially distributed to each embryo cup and the process repeated for at least two more substrates until the proper number of embryos have been placed in each cup to give at least 50 embryos per treatment. The embryo cups should be standing in dilution water when the embryos are being distributed and then the cups should be randomly placed in the test chambers.

17. Twenty-four hours after they are placed in the embryo cups, the embryos should be visually examined under a dissecting scope or magnifying viewer and all dead embryos discarded. Embryos that are alive but heavily fungused should also be discarded. Forty-eight hours after the start of the exposure, all dead and heavily fungused embryos should be removed and the remaining healthy, fertile embryos randomly reduced to the required number (at least 30 per treatment). If more than about 25 percent of embryos in the control treatment are discarded within the first 48 hours of the test because they are dead or heavily fungused, the test should probably be restarted. Each day thereafter dead embryos should be discarded.

18. In each treatment, when hatching is about 90 percent completed or 48 hours after first hatch in that treatment, the live young fish should be counted and released into the test chambers. Unhatched embryos should be left in the cups and released into the test chamber when they hatch. The range of time to hatch in each cup should be recorded.

19. A test should be terminated if the average percent hatch in any control treatment is less than 50 percent or if the percent hatch in any control embryo cup is more than 1.6 times that in another control embryo cup.

20. The flow rate, size of the test chamber and the amount of food added should be such that the average weight of the controls at the end of the test would not be significantly greater if only half as many fish were tested.

21. Each test chamber containing live fish over 2 days old must be fed live, newly-hatched brine shrimp at least twice a day at least 6 hours apart (or three times a day about 4 hours apart) on days 2 to 5 after hatch and at least 5 days a week thereafter. They must be fed at least once a day on all other days. Other food may also be provided in addition to the above. The amount of food provided to each chamber may be proportional to the number and size of fish in the chamber, but each chamber must be treated in a comparable manner. Quantifying the amount of live, newly-hatched brine shrimp to be fed is difficult, but the fish should not be overfed or underfed too much. A large buildup of food on the bottom of the chamber is a sign of overfeeding. A sign of not feeding enough of the right kind of food is that in a sideview the abdomen does not protrude.

22. Test chambers should be cleaned often enough to maintain the dissolved oxygen concentration (see items 11 and 12) and to ensure that the toxicant concentrations are not decreased significantly due to sorption by matter on the bottom and sides. In most tests if the organisms are not overfed too much and the flow rate is not too low, removing debris from the bottom once or twice a week should be adequate. With some toxicants that promote growth of bacteria, the sides and bottoms should be cleaned more often. Debris can be removed with a large pipette and rubber bulb or by siphoning into a white bucket. The pipette or bucket should be examined to ensure that no live fish is discarded.

23. Temperatures should be recorded in all test chambers once at the beginning of the test and once near the middle of the test. In addition, temperatures should be recorded at least hourly in one test chamber throughout the test.

The dissolved oxygen concentration should be measured in each treatment near the 1st, 21st and 28th days of the test.

Hardness, pH, alkalinity, and acidity should be measured once a week in the control treatment and once in the highest toxicant concentration.

The concentration of toxicant should be measured at least twice a week in each treatment.

24. Dead fish should be removed and recorded when observed. At a minimum, live fish should be counted 11, 18, 25 and 32 days after the beginning of the test. The fish should not be fed for the last 24 hours prior to termination. At termination the number of fish that are visibly (without the use of dissecting scope or magnifying viewer)

grossly abnormal in either swimming behavior or physical appearance should be determined. Also at termination the weight (wet, blotted dry) of each fish that was alive at the end of the test should be determined. If the fish exposed to toxicant appear to be edematous compared to control fish, determination of dry rather than wet weight is desirable.

25. A test is not acceptable if the average survival of the controls at the end of the test is less than 80 percent or if survival in any control chamber is less than 70 percent.

26. A test is not acceptable if the relative standard deviation (RSD = 100 times the standard deviation divided by the mean) of the weights of the fish that were alive at the end of the test in any control test chamber is greater than 40 percent.

27. Data to be statistically analyzed are:

- Percent normal hatch
- Percent survival at end of test (based on fry, not embryos)
- Percent normal at end of test (based on fry, not embryos)
- Weights of individual fish that were alive at end of test.

28. Dichotomous data (live-dead, normal-abnormal) should be analyzed using contingency tables or log linear techniques. For weight data, the individual fish are used as the replicates unless a two-tailed F-test indicates that differences between replicate test chambers are not negligible. Weight data may be analyzed using Bartletts' test and one-way analysis of variance, but to obtain information concerning the upper and lower chronic values, Dunnett's procedure (Steel and Torrie, Principles and Procedures of Statistics, 1960, p. 111) should be used to identify treatment means that are statistically significantly different from the controls at the 95 percent level.

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This tentative procedure was written by Charles Stephan with the help of many members of the staff of the Environmental Research Laboratory in Duluth, Minnesota.

EFFLUENT GUIDELINES LIMITATIONS AND LETHAL UNITS

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I've been asked to talk a little bit about the toxic unit concept. It seems to me, from some of the questions that have been asked, that there still seems to be a certain amount of confusion in the minds of some people here about what exactly this is all about and why you should be concerned about it. Therefore, I'll take just a minute to review a few concepts that led me to go through some of the thought processes which I did in preparing this paper.

Obviously, the requirement for measuring toxicity is here to stay whether people in the agency who have to deal with the problem or people in the industry who have to live with the problem like it or not. Another thing that's just as clear is that the reliance on analytical chemistry measurements in the permit program has not adequately addressed the question of toxicity, primarily because constitutents not routinely monitored can, and do, contribute to toxicity. However, I think it's also obvious that increasing the analytical burden of the permittee is not really the answer, despite the fact that you might feel you'd rather make a few more measurements than deal with this nebulous area called "biomonitoring". I think in the long run, as Dr. Mount suggested this morning, we'll find that the cost and the effort related to doing it biologically and toxicologically are probably going to be less than continuing to increase the already complex and significant analytical costs.

But more important are the toxicological reasons why analytical measurements aren't the answer. Even if we did measure everything that was in the waste, we'd have to know the toxicity to the aquatic organisms of each of those constituents. If any of you have looked at the proposed water quality criteria documents being published under the consent decree, you'll find that even for some very, very common compounds like benzene and chloroform, we don't have toxicity data for aquatic organisms. As the chemicals in your waste become more and more unique or more and more characteristic of a particular industry, the less and less likely it is that such data exists.

Furthermore, even if we had such data, we would then need data on all of the combinations of constituents that could be in the wastewater and the interactions toxicologically that could occur there. So it's clear that measuring all of these constituents is not the answer - we can't do it, we don't have half of the toxicity data required, and even if we did, we would still have a long way to go. So the simplest approach is to measure the toxicity of the whole waste, and that is what I think this meeting is all about.

I'll spend a little bit of time talking about toxic units although I'm not sure in my own mind where we can go with it at this point. Back in 1971, a group working in the San Francisco Bay area led by Esvelt and coworkers at the University of California completed a comprehensive study of the toxicity of municipal wastes to aquatic organisms. As a result of these efforts they proposed measuring, monitoring, and regulating toxicity as we have historically regulated conventional pollutants such as solids, BOD and COD. In its most simplistic form, they proposed to measure toxicity, establish a discharge limitation in the form of a mass emission rate, and apply treatment technology designed to "remove toxicity" in order to meet the reasonably established limitation.

I guess I better go into a little detail here because this toxic unit concept can be confusing if you try to make it anything more than it is. All it represents is a simple alternative way of expressing an LC50.

Basically what they proposed was that LC50 values, expressed normally as mass/volume or volume/volume concentrations, be translated into what would represent a relative toxicity "concentration" which could be expressed as toxic units. The concept of toxic units is well established in the literature at this time. In its simplest form, if you have a constituent like copper whose LC50 to some organism is 2 μg/1, and you have 10 μg/1 of copper in your waste, the copper is said to contribute 5 toxic units to the toxicity of the waste. When you have an effluent, for which the effective concentration of the "constituent" is 100 percent, the number of toxic units in the effluent is simply the LC50 for that effluent to some organism divided by 100. The quotient thus represents a unitless measure of toxicity arbitrarily called the toxicity "concentration" and expressed as toxic units. Unfortunately, the use of the term concentration can be misleading as the calculation yields toxic units, a measure independent of any specific volume - it's 2 per liter, 2 per gallon, 2 per million gallons, whatever. They further propose, with respect to the mass emission rate, that the product of the toxicity concentration (in toxic units per some unit volume) in the wastewater times the flow rate of the wastewater in the same unit volume per unit time would yield a mass emission rate in toxic units per unit time.

The authors recognized that, if toxicity was to be considered in water quality management in a manner similar to conventional pollutants, they had to evaluate the effectiveness of conventional and advanced treatment technologies to reduce toxicity. They also recognized that, since a toxic unit was a test-specific parameter, the tests had to be fairly standardized and reproducible. This latter point is particularly important if, in fact, the concept of toxic units ever gets into the NPDES program. I'll elaborate on that in a few minutes.

I'd like to give you just a brief outline of the results of Esvelt's program. They worked primarily with five municipal waste effluents, and the mean LC50 value of all samples tested ranged from 40 to 60 percent effluent. These LC50 values translate to 1.5 to 2.5 toxic units. Furthermore, they found that the mean relative standard deviation derived from repeating tests 24 times on the same source of effluent was about ±20 percent. Neither of the above findings should be particularly startling, as relatively low toxicity and relatively little variability in the toxicity of municipal waste would not be unexpected.

They went on to point out that when they evaluated the mass emission rate for a single effluent tested daily for eight consecutive days, they found that, although the absolute LC50 value and the absolute flow rates could vary by as much as 100 percent, the mean mass emission rate for toxicity had a relative standard deviation of only 15 percent.

Unfortunately they didn't go on to say whether this was coincidence or whether they had some fundamental basis for assuming there is a real relationship here. I certainly don't know what the relationship would be, and I suspect it's more coincidence.

They went on to look at the reproducibility of these toxicity tests in their program, and they found that the replication of results was excellent with a relative standard deviation of only 5 percent. Now that's a lot better than I've ever done, and I suspect it's a lot better than most other people in the room have done. Again, I don't know what the reasons for those numbers are, but in my opinion I believe a more representative number of reproducibility would be something on the order of 15 to 20 percent.

They also went on to look at whether one could treat toxicity to reduce it. They looked at biological treatment as you would in a municipal waste system, and they found that even for those wastes that were relatively non-toxic to begin with (1 to 2 toxic units), biological treatment reduced what little toxicity was there by more than 75 percent.

Now that's all I'm going to say about the toxic unit concept. It's just another way of expressing the toxicity which one measures by a bioassay. I'm a little concerned about using it in a mass emission sense because, even in this limited program, they showed that the same waste could vary by ± 20 percent. If you believe that the reproducibility of the bioassay is ± 20 percent, then you've got a propagation of errors there that makes it very hard to live with a single mass emission rate number.

Now I'd like to tell you about some studies that we conducted for EPA to further evaluate this toxic unit concept and how it might apply to industrial waste treatment facilities and NPDES programs.

Based on the results of the study by Esvelt and his coworkers, and motivated by a desire to control toxicity of wastewaters discharged into surface waters, the EPA was concerned with answering several questions about this potential approach. The questions to be answered in our program were concerned with (1) the efficacy of treatment systems for removing toxicity in industrial wastes; (2) the variability of the toxicity of both the influent to and the effluent from industrial waste treatment systems; (3) the required frequency of sampling for enforcement and compliance monitoring; and (4) the effect of species selection for monitoring. EG&G, Bionomics, using mobile laboratory facilities, visited seven industrial sites (comprised of one organic chemical facility, five pesticide facilities - selected because of their anticipated relatively high toxicity, and one pulp and paper facility). The sites were also selected because they represented various types of treatment technologies essentially equivalent to BPT, and in some cases technologies more advanced than BPT. In order to investigate some of those areas of interest to the agency, we conducted 24-hour static toxicity tests with fathead minnows using batch wastewater samples collected twice a day for 10 consecutive days. Also, in order to investigate the effect of species selection, we concurrently tested Daphnia magna on alternate days. And I'd like now to show you some of that data.

The first data set is the results, expressed in toxic units, of tests with fathead minnows at a pulp mill of International Paper Company in Georgetown, South Carolina (Table 1). There are several things to note about these data. First is the relatively low toxicity of the influent to the treatment system. Second, and perhaps more important, is the relatively low variability in the toxicity of the influent to the treatment system. The third is the complete removal of toxicity by the treatment system. Lastly, we find that the comparability of the results from the two species tested seems reasonable. Now this is obviously a pretty clean plant.

The next data set is from an organic chemicals plant, Union Carbide's plant in Charleston, West Virginia (Table 2). Significant aspects of these data are the slightly higher toxicity of the influent; the occasional order of magnitude excursions in toxicity of the influent; the complete removal of toxicity as a result of treatment even in the face of these relatively large excursions; and again the comparability of the daphnid data to the fathead minnow and the ability of either species to detect the rather large excursions in the toxicity in the influent. In this case the treatment system did not entirely remove the toxicity, although I must caution you that measurements of toxic units less than unity are tentative to say the least. You have to extrapolate when you don't have a complete 100 percent mortality or mortality above 50 percent to try to get at these numbers, and I wouldn't put a lot stock in toxic units less than unity.

The last set of data is from a pesticide manufacturing plant operated by Diamond Shamrock in Green Bayou, Texas (Table 3). This plant is characterized by a relatively high variability in the toxicity of the influent to the treatment system, with excursions exceeding two orders of magnitude; on the other hand you get absolutely zero variability in the effluent from the treatment system. So there is a rather remarkably consistent degree of

Table 1

TOXIC UNIT CONCENTRATION IN THE EFFLUENT TO, AND EFFLUENT FROM THE INTERNATIONAL PAPER COMPANY INDUSTRIAL WASTE TREATMENT PLANT GEORGETOWN, SOUTH CAROLINA

Sample	Minnow		Daphnid	
	Influent	Effluent	Influent	Effluent
AM-1	1.9	0	<1.5	0
PM-1	2.9	0	2.0	0
AM-2	2.4	0	-	-
PM-2	2.9	0	-	-
AM-3	2.9	0	1.8	0
PM-3	2.4	0	1.2	0
AM-4	2.4	0	-	-
PM-4	1.9	-	-	-
AM-5	1.9	0	1.3	0
PM-5	2.4	0	1.4	0
AM-6	2.4	0	-	-
PM-6	4.3	0	-	-
AM-7	2.4	0	1.3	0
PM-7	2.4	0	<1.5	0
AM-8	4.3	0	-	-
PM-8	4.8	0	-	-
AM-9	2.4	0	2.4	0
PM-9	2.9	0	2.4	0
AM-10	3.7	0	-	-
PM-10	>8.3	0	-	-
Mean	2.8		1.7	
(S.D.)	0.8		0.5	

Table 2

TOXIC UNIT CONCENTRATION IN THE EFFLUENT TO, AND EFFLUENT FROM THE UNION CARBIDE INDUSTRIAL WASTE TREATMENT PLANT

CHARLESTON, WEST VIRGINIA

Sample	Minnow		Daphnid	
	Influent	Effluent	Influent	Effluent
AM-1	6.7	0	4.8	0
PM-1	10.5	0	5.9	0
AM-2	4.3	0	-	-
PM-2	5.6	0	-	-
AM-3	6.7	0	3.8	0
PM-3	8.3	0	4.8	0
AM-4	4.0	0	-	-
PM-4	6.7	0	-	-
AM-5	3.6	0	5.3	0
PM-5	3.6	0	7.1	0.6
AM-6	4.5	0	-	-
PM-6	7.7	0	-	-
AM-7	50.0	0	55.6	0
PM-7	6.3	0	7.7	0.6
AM-8	13.3	0	-	-
PM-8	8.3	0	-	-
AM-9	4.5	0	2.0	0.6
PM-9	38.5	0	21.3	0.8
AM-10	3.6	0	-	-
PM-10	5.6	0	-	-
Mean	10.2		11.8	
(S.D.)	11.7		15.4	

Table 3

TOXIC UNIT CONCENTRATION IN THE EFFLUENT TO, AND EFFLUENT FROM THE DIAMOND SHAMROCK INDUSTRIAL WASTE TREATMENT PLANT GREEN BAYOU, TEXAS

Sample	Minnow Influent	Minnow Effluent	Daphnid Influent	Daphnid Effluent
AM-1	>400	1.2	535	3.1
PM-1	794	1.2	>1700	2.5
AM-2	37	1.2	-	-
PM-2	952	1.2	-	-
AM-3	19	1.1	-	-
PM-3	20	1.1	-	-
AM-4	47	1.2	-	-
PM-4	>2000	1.1	-	-
AM-5	12	1.2	26	2.5
PM-5	19	1.1	42	2.5
AM-6	400	1.1	-	-
PM-6	24	1.1	-	-
AM-7	30	1.2	96	2.5
PM-7	24	1.2	71	3.1
AM-8	1785	1.2	-	-
PM-8	1785	1.2	-	-
AM-9	19	1.2	24	2.5
PM-9	24	1.2	28	2.5
AM-10	>2000	1.2	-	-
PM-10	588	1.2	-	-
Mean	373	1.2	133	2.6
(S.D.)	645	0.05	199	0.25

removal of toxicity even in the face of unusually high excursions. Also, these data again suggest that if you had to decide whether to use one species or the other, I don't really think it makes much difference. Certainly from an economy of scale, the Daphnia would seem to be a much more attractive organism.

Now very quickly some other data sets were very similar. For example, the treatment system at a pesticide plant operated by Monsanto in Muscatine, Iowa removed an average of 96 to 98 percent of the toxicity of the waste going into the system. And the mean toxic unit concentration of the effluent for the treatment system for fatheads was 1.5 ± 0.3, and for daphids it was 1.3 ± 0.2 - essentially the same numbers.

The treatment system at Monsanto's pesticide plant in Luling, LA removed 92 percent of the toxicity, leaving an average of about 6.0 toxic units.

Now, what can we do with all this information? I'd like to take just a few minutes to give you my thoughts. Let me preface these comments by emphasizing the fact that the following thoughts are my own and should not be construed as representing in anyway the conclusions or philosophy of the agency, nor any of the companies participating in the project.

It seems to me that data on the toxicity and variability of influent to a treatment system should be considered by the industry in the same light as any engineering data for the design and operation of a treatment system. Similarly, such data should be of interest to the agency only as it relates to an assessment of the technological capability to remove toxicity - that is, is it feasible, is it practical, is it economical, and so forth.

It further seems to me that, from the standpoint of regulating toxicity, it is the data regarding the toxicity of the effluent from the treatment system which should be of prime consideration. With that in mind I'd like to take a look at a summary of these data (Table 4). These are the mean number of toxic units in the plant effluents which we evaluated to both fathead minnow and daphnid. The important thing to note here is the relative standard deviation of the mean. You can see it runs as high as 26% in one case and as low as 4 percent in another, but that's fairly uncharacteristic. A value between 10 and 20 percent is a good number. These data suggest that the toxicity of discharges from treatment systems does not vary dramatically when the system is up and functioning and that approximately 95 percent of the time a bioassay should produce results within 25 to 50 percent of a mean value, assuming a level of reproducibility in bioassays of 20 percent. This observation relates to the toxic unit limitation. It appears that any toxicity limitation, or "lethality baseline", included in a permit would of necessity have to be incorporated in the form of a "range" of acceptable values. I don't see how you could pick a single number and say "thou shalt not ever violate it". The data further suggests that perhaps one could expect slightly less variability using daphnids, although I'm not sure those differences are significant. I personally believe that the utility of toxicity tests lies in providing data for establishing the permit conditions

Table 4

MEAN (RELATIVE STANDARD DEVIATION, %) TOXIC UNIT CONCENTRATION IN THE EFFLUENT FROM INDUSTRIAL WASTE TREATMENT SYSTEMS

PLANT	TYPE	MINNOW	DAPHNID
Georgetown, SC	Paper	0	0
Charlestown, WV	Organics	0	0
Muscatine, IA	Pesticide	1.5 (20%)	1.3 (15%)
Green Bayou, TX	Pesticide	1.2 (4%)	2.6 (10%)
Kansas City, MO	Pesticide	3.8 (26%)	4.4 (9%)
Luling, LA	Pesticide	6.0 (23%)	3.2 (22%)
Laporie, TX	Pesticide	2.4 (12%)	-

relative to toxicity. However, I have serious questions about the utility of such tests for monitoring compliance with those permit conditions. Now let me go on to explain this last statement.

I think that bioassays could provide significant information on the efficacy of treatment systems for removing toxicity. Clearly they can provide information on what I call the "residual acute toxicity" of the wastewater and on normal variability in toxicity of a typical discharge remaining after treatment, thus providing relevant information regarding dilution required in a stream to meet water quality standards. I think a program similar to the ones we've described here would provide data on the small and random fluctuations in effluent quality associated with the regular operation of an industrial plant and its effluent control equipment, thus defining a range of LC50 values which might be considered "normal". I think if you couple this approach with the embryo-larval test that Dr. Mount described earlier, one could even use bioassays to evaluate the potential hazard associated with the long term chronic effects of "residual toxicity" of a typical discharge after treatment on receiving water organisms.

I strongly urge both the agency and industry to utilize reliable toxicity testing with aquatic organisms, where appropriate for toxicologically characterizing a waste stream for the purpose of establishing permit conditions with regard to toxicity, and the required degree of control of toxicity.

However, I do have some problem with the concept of requiring industry to use bioassays as a monitoring tool. An effective monitoring tool should be one which has the capacity to detect most or all excessive pollutant discharges to the environment. Now clearly bioassays have the capacity to do this as demonstrated by some of the data we've just looked at. However, the monitoring system should produce information on three critical variables: the frequency, intensity, and duration of significantly elevated pollutant discharges. Now although bioassays have the ability to detect the intensity, they don't tell us much about the frequency or the duration. I believe that bioassays could provide such information if utilized frequently enough. However, the costs of using bioassays with sufficient frequency (real-time, hourly, or daily) would clearly be prohibitive, and I think such monitoring could be more effectively done using a surrogate physical or chemical parameter for which changes can be correlated with, or indicative of, changes in toxicity.

For example, there is some data that suggests that with certain wastes there is a straight correlation between the pH of the waste as it comes out of the treatment system and toxicity. There may be a future in using something like a Microtox system which can be correlated to toxicity as a routine monitoring tool. Now the reason I say this is because of the frequency with which I think we'd have to do bioassays to effectively monitor effluent toxicity. I base this observation on a recently published study on a paper mill in British Columbia by Nemetz and Drechsler. This represents the most complete and comprehensive analysis of sampling design in effluent monitoring which I've ever seen. In it they concluded that "composite sampling even at frequent intervals...performs the ironic function of apparently measuring elevated pollutant releases without detecting them." As a result, they conclude that composite sampling fails to provide either of the essential functions of an effective monitoring system: (1) a high representational accuracy of the effluent profile; or (2) the detection of most or all pollutant releases with potential serious environmental consequences. They recommend that various considerations be given to the cessation of composite sampling in effluent monitoring programs and their replacement by frequent grab sampling and sequential analysis. How frequent is the question. They found that with a paper mill where they were measuring a parameter having a mean of X, with an N of 120,000, and a relative standard deviation of 50 percent (similar to bioassay data) that the duration of 95 percent of the "spills" was less than 4 hours. As I interpret their data, they further estimated that if one took instantaneous grab samples every day, one would still not detect 99 out of every 100 "spills". Clearly then, monitoring using bioassays is not a feasible method of "monitoring" toxicity. I'm not suggesting that bioassays have no place during the life of a permit. I'm clearly suggesting that their place is in the establishment of the permit conditions. But in addition, if one has evidence to suggest that, or expect that, the quality of the discharge may be significantly different from the typical discharge, either due to changes in the inputs to the system or in the operation of the system, it might be prudent to monitor the toxicity of the discharges using bioassays at these times. However, I see little utility in arbitrarily predetermined, periodic (e.g., quarterly or annual) toxicity testing except to reassure everyone that the system is working as it should on the day and at the time the sample was taken.

APPLICABILITY OF THE AMES TEST IN BIOMONITORING

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Most of the things that you've heard so far deal with assays that detect mortality, and/or morbidity in different test organisms. This assay that we'll be describing detects changes that occur in hereditary material. In other words, the question that we'll be asking is: Do the chemicals in effluents that are tested alone, when reaching hereditary material, cause changes in the hereditary material that can be inherited? Do chemical effluents have the capability of causing hereditary diseases or if interactions occur in what are called somatic cells of the body, cells other than the gonadal tissue cells, could this interaction possibly cause cancer?

The test that we'll be describing today is commonly called, and most people know of it as, the Ames test. It's used for a variety of reasons. There are several reasons why people might like to use it. It is rapid and inexpensive. It is a first level indication of genotoxicity rather than mortality or morbidity, and we can use it to set priorities in further genotoxicity testing. Something that's very important to many of you is that we can use this test coupled with chemistry, technology, and a variety of other things in order to direct the development of technology and to help those who are in technology development to know what types of active compounds are coming out of the system.

There are two assumptions I think that you should be aware of that this test is based upon. First, we are assuming, and this is generally accepted in the scientific world today, that the structure of DNA, not chromosomes, but DNA structure, is the same for all organisms, except for some viruses. Second, gene mutations that occur at the DNA level will occur generally by the same molecular mechanisms and therefore be inherited in the same way. There are some debatable issues within that.

The Ames test is a test run with a bacterium called Salmonella typhimurium. Figure 1 is an outline of how the test is run. It's a technically simple test to perform; however, it gets more complicated when you get into the laboratory. Essentially what we do is we have a soft agar overlay that is melted. Into this overlay we add the bacteria indicator organism, the chemical or substance or effluent that is to be tested, and we, on occasions, will also add in a mammalian activation system. We refer to this activation system as S9 because it is a 9,000G supernatant microsome-containing fraction. This is used to activate promutagens and procarcinogens to an active form. After these are combined together into the agar overlay and overlaid onto the plate, they are incubated for 48 hours. These are derived bacterial strains with a mutation within the histidine locus. They mutate "in reverse" to the wild type, which means that they can grow in the selective media and when that reversion occurs we get a response. We plate approximately 10^6 bacteria per plate; therefore, on a control plate we'll find a few spontaneous mutant colonies that will grow. If our substance is mutagenic and has no killing, we'll see increasing numbers of colonies with increasing dosage. So therefore, we tested a variety of doses; we usually do this in our laboratory in triplicate at each dose. We run the proper positive and negative controls and usually, if we have enough material, replicate the experiment. So we get a fairly large amount of data coming out of this.

Bruce Ames in developing this system recognized, as most geneticists do today, that there is a variety of gene mutations that can occur; therefore, he developed five different tester strains of bacteria. Each tester strain has a specific type of mutation incorporated and other characteristics that are important to the people conducting the test. But generally, for a complete screening, at least these five strains and sometimes other strains, if you're in a research mode, could be used.

There are some major variables that can occur within a system. We are maintaining a biological organism over time, frozen, and yet even with that there are some natural variations within subcultures. This makes the use of positive and negative controls, the checks that Ames describes for his strains, mandatory for essentially every test. There are also areas of variation within the mammalian activation system, but the greatest variation that's brought into the test is usually due to the physical and chemical characteristics of the substance that's to be tested.

One of the topics I was asked to present is the equipment that's generally needed. Table 1 is a list of this equipment. A laminar flow biological safety hood is needed because we do use known carcinogens and mutagens which are hazardous to humans and the safety factors within the laboratory are very stringent. Much of the remaining list also involves safety. We allow no mouth pipetting, the area has to be properly exhausted, and so forth. This can be quite expensive. These are optional types of equipment that you can use which would increase the use of your manpower, and there is other common biological equipment that might be needed.

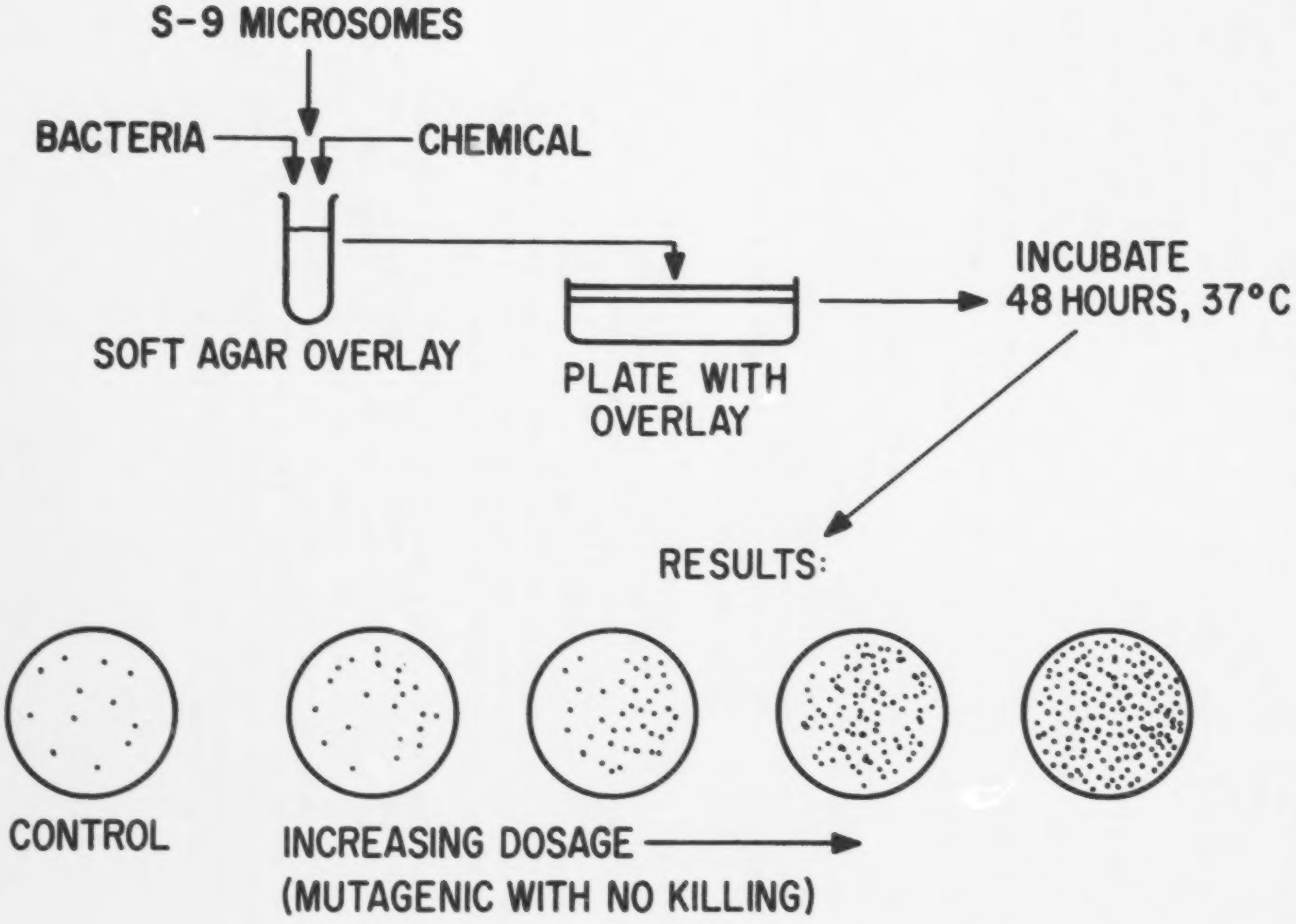
S-9 MICROSOMES
BACTERIA
CHEMICAL
SOFT AGAR OVERLAY
PLATE WITH OVERLAY
INCUBATE 48 HOURS, 37°C
RESULTS:
CONTROL
INCREASING DOSAGE
(MUTAGENIC WITH NO KILLING)

Figure 1. Schematic diagram of the plate incorporation (Ames) test.

Table 1

EQUIPMENT

- Laminar Flow Biological Safety Hood
- Automatic Pipets
- Properly Exhausted Incubators and Work Areas
- (Automatic Colony Counter)
- (Automatic Plate Pourer)
- Other Equipment Common to Microbiological Laboratory

Two things that I forgot to add onto this list are very important. Most people prepare their own mammalian microsomal activation, and so if you do that, you'll have to plan also on having a rodent colony and biochemical laboratory backup to check those preparations.

When it gets to you, you're probably not interested as much in the first part as in how we interpret the data that's coming out of the Ames test. I'd like to run very quickly through some aspects that are generally accepted with the Ames test. A positive response (Table 2) is usually easy to ascertain because positive responses are usually dramatic in the vast majority of cases that we've dealt with. A positive dose is defined in the following manner: There's at least a 2.5 fold increase in revertant colony counts over the spontaneous or control level. When you plot the dose response from the zero dose up to your highest dose, there's usually a very regular type of dose response curve. As I mentioned, we always run specific controls for these particular strains. We expect the proper response in each of those controls. Another thing that we can do in the system is that after we find some mutants, in order to make sure that we're not getting a false positive, we can check these mutant colonies to make sure that they truly are mutants. So, this is what we would define as a definite positive response.

Table 2

DATA INTERPRETATION

Positive Response

- At Least 2.5 x Increase
- "Regular" Dose Response Curve
- Expected Response for all Controls
- Check for True Reversion

One of the somewhat more difficult jobs, however, is assigning a negative response. Table 3 gives the criteria that we use in our laboratory for assigning a negative response. This may vary a little bit according to whom you talk to, but this is pretty acceptable now to most people. We expect, within our laboratory, at least five doses at 1/2-log intervals. So we're exploring quite a dose range when we do the test. The top dose we expect to be either toxic or greater than or equal to 5 mg per plate. This amount is chosen because it's physically about the largest amount that you could normally put into the test. We also expect the proper responses from all controls, of course. We expect the doses to be replicated within the experiment or we expect replicate experiments - either would be acceptable. And we expect the major tester strains to be used and the proper sterility controls to be completed. If you find that all of these components are completed and you have no dose response -- there's no activity other than spontaneous -- you can be confident in saying that the Ames test indication is negative.

Table 3

DATA INTERPRETATION

Assigning A Negative Response:

- At Least 5 Doses At Half-Log Invervals
- Top Dose is Toxic or Equals or Greater than 5 mg/Plate
- Expected Responses from all the Proper Controls
- At Least Replicate Dose Responses and/or Experiments
- Tested in 5 Major Tester Strains
- Proper Sterility Controls

That leaves a little bit of data that's left with a questionable response (Table 4). Sometimes you'll see an increased response with dose but it doesn't ever reach the 2.5 fold level. Sometimes the response will approach that point. Because of the toxicity that tends to mask the response after that point, you can't force the response to go past a 2.5 fold increase. So this leaves you with a questionable result. This uncertainty can usually be removed by replicate experiments. Sometimes you will see at certain doses that, indeed, you have a count that's greater than a 2.5 fold increase, but the data is very irregular. We would generally call this a negative, qualify, and explain it.

Table 4

DATA INTERPRETATION

Questionable Responses:

1. Increase Response With Dose, But Never Exceeds Spontaneous Level by 2.5 Fold
2. Irregular Response Curve With One or More Responses Greater Than 2.5 x Spontaneous

There are many different ways, and Table 5 lists some of the ways, that you may see the data summarized. When you get out the data from one experiment in the Ames test, you're looking at many strains of bacteria done in triplicate at five doses or more each. Then with a whole series of controls, you're looking at over 200 pieces of data in one test; and we can run three to four tests a week in one laboratory with just two technicians. So there's a vast amount of data, and people, including us, tend to summarize data in a variety of ways. So there are a variety of ways to summarize data; however, we tend to be very careful in comparing data by using only specific activity or a statistical modeling curve.

Table 5

DATA INTERPRETATION

Methods of Data Summary:

1. Fold Increase or Mutagenic Index
2. Net Response (In Revertants Per Plate)
3. Specific Activity
4. Specific Activity Fold Increase
5. Specific Response Activity
6. Maximum Response
7. Statistical Modeling Curve

For those who are involved in water effluents, there are some associated techniques (Table 6) that turn out to be very important: concentration, extraction, and fractionation. There are a variety of concentration procedures, and most of you are probably quite familiar with these. And then there are extraction procedures. Within water samples from effluent sites you also find particulates, and this is where extraction can become very important. Particulate matter generally interferes with the test. Particles make plates very hard to read, to count, and so forth. So generally what we do is extract the organics off the particulate matter by different methods and then place the extract into the test system. Fractionation can be important if you're going into technology development and want to know what compounds or what types of compounds are causing the activity.

Table 6

ASSOCIATED TECHNOLOGIES

1. Concentration
    A. Adsorbants (XAD)
    B. Lyophilization
    C. Reverse Osmosis
    D. Filtration (Particulate)
    E. Extraction

2. Extraction
    A. Solvent Systems
    B. Sonication
    C. Physiological

3. Fractionation

There is a variety of other tests that I'll just review very quickly that are essentially modifications of the Ames test - the original Ames test. One is a simple spot test (Figure 2) where the bacteria and activation system are first put on a plate, the substance is spotted in the center, and you get either a negative or a positive in which you had a zone of killing and a zone of mutation and outside of that another zone of spontaneous mutation. For many of the things that you'll be dealing with, this will not work too well. Many of the organics that you test will not diffuse readily in agar, and so the spot test has limited applicability when you concentrate the organics.

There's a liquid suspension test (Figure 3) which is important because it not only gives you a revertant number, but it also gives you the survival number. For a geneticist this can turn out to be very important because he's looking at the number of hits on a particular gene in a particular organism. Very seldom, unless you are in a research mode, would you want to use this test. There are a couple of occasions, however, if you're working with some specific types of compounds such as nitrosamines, when you may want to go to a test such as this.

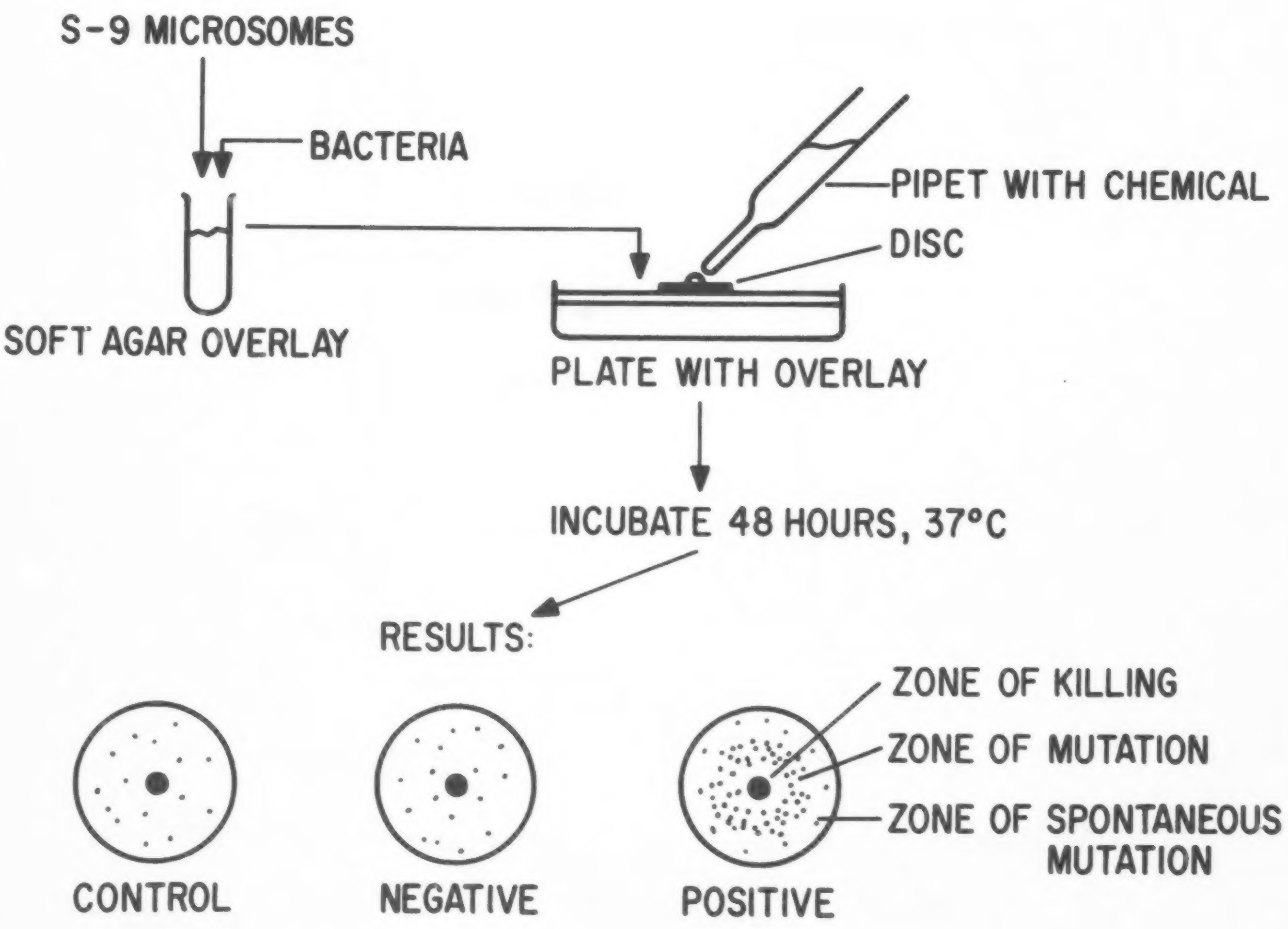
S-9 MICROSOMES
BACTERIA
SOFT AGAR OVERLAY
PIPET WITH CHEMICAL
DISC
PLATE WITH OVERLAY
INCUBATE 48 HOURS, 37°C
RESULTS:
ZONE OF KILLING
ZONE OF MUTATION
ZONE OF SPONTANEOUS MUTATION
CONTROL
NEGATIVE
POSITIVE

Figure 2. Schematic diagram of the spot test.

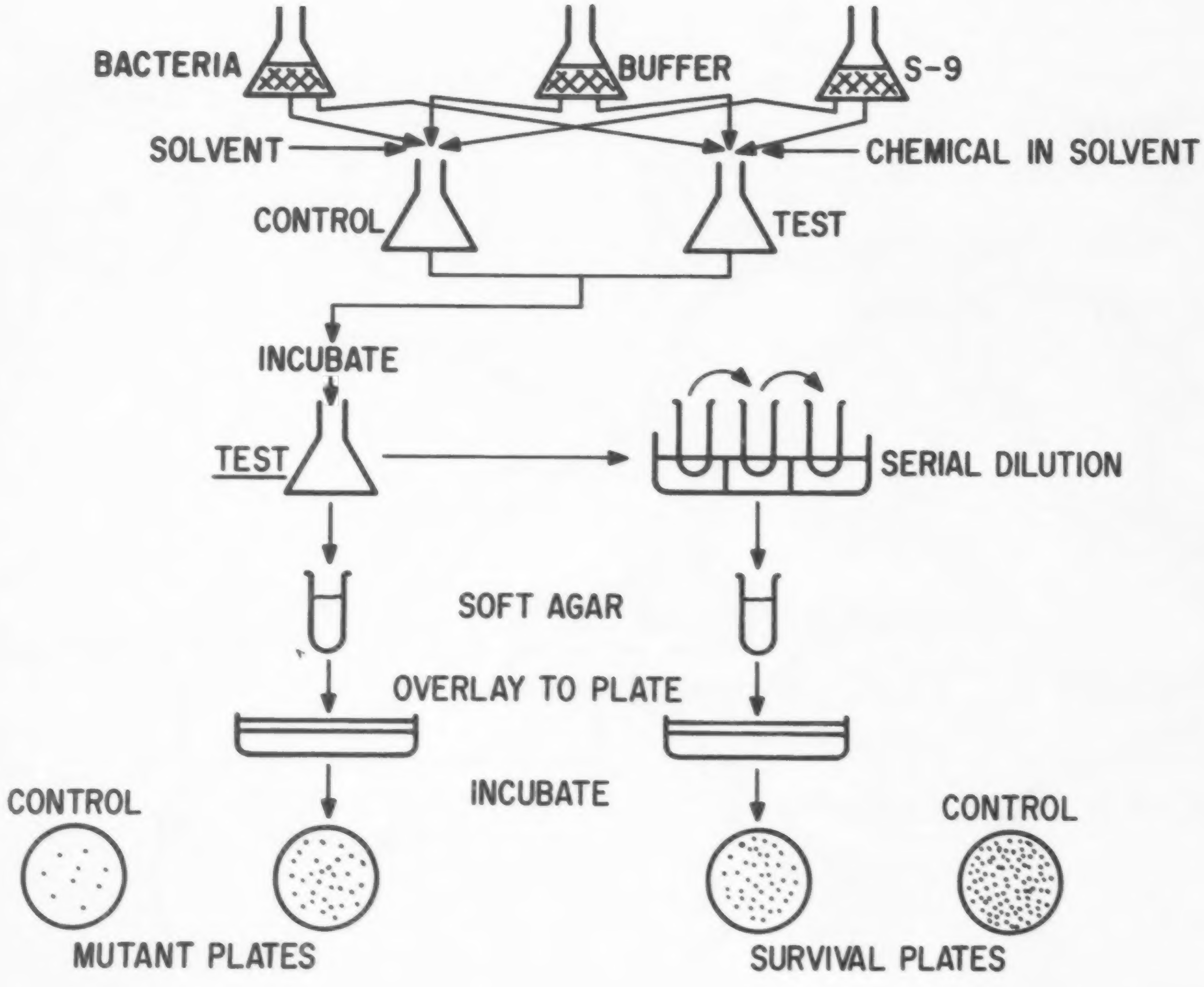
BACTERIA
BUFFER
S-9
SOLVENT
CHEMICAL IN SOLVENT
CONTROL
TEST
INCUBATE
TEST
SERIAL DILUTION
SOFT AGAR
OVERLAY TO PLATE
INCUBATE
CONTROL
CONTROL
MUTANT PLATES
SURVIVAL PLATES

Figure 3. Schematic diagram of the liquid suspension test.

Something that's very similar but more rapid than the suspension test is the preincubation test (Figure 4) which is just a shortened modification of the suspension test. It is also good for testing compounds such as the nitrosamines.

A modification that we've developed to the spot test is the well test (Figure 5) in which we can test two chemicals - a solvent control and a positive control - with and without activation on one plate.

I've run through quite a bit very rapidly - it's really about a 2 hour lecture what I've tried to do in about 20 minutes here. I brought along two articles that you might be interested in. One of them describes the Ames test in more detail - some of the pitfalls and some of the uses of the Ames test. The other is a much more general and lay-like publication that describes short term tests for carcinogens, mutagens, and other genotoxic agents.

DISCUSSION

Question: I am a plant engineer of a medium sized steel plant on the shore of Lake Erie at Lorraine. I am impressed by the enthusiasm and great mental capacity devoted to the goal here of preserving the long term existence of aquatic organisms. From where I sit, I am also interested in preserving the existence of an organism. The organism I think of is my plant. It employs about 7,000 to 8,000 people; about 30 to 40 percent of the members of the community depend on its employment. And gentlemen, that organism finds itself in a heavily polluted environment. What this pollution is and who the polluters are of this environment that makes it so difficult for these organisms to survive, I leave to everybody's imagination here. I can take some inflation, taxation, and here comes the 30-day regulation. I was one of the unfortunate cosignatories of a $500,000 order a few months ago, and that money is going to give us nothing but a book or maybe two books that went to a consulting firm to tell us just what this plant, this medium sized steel plant, would have to do to comply with BAT. Now we've been through BPT, we've been through NPDES, we've been through air problems, and now we are facing BAT. I want to be sure that now that you've invited me to this conference, when I get back I get the right message to my plant management and to the corporate staff that we work with, and it poses a little question to me. In a few months we are going to get out a few nice little books which will say this is what we'll offer to the regulating agencies as our BAT approach. Now, my question to you gentlemen is, in developing your emerging science of the effects of toxicity on aquatic organisms, are you going to give us your virginity - the industry your virginity - when BAT time and negotiations and decisions come around to land on something that meets the long term requirements economically. My

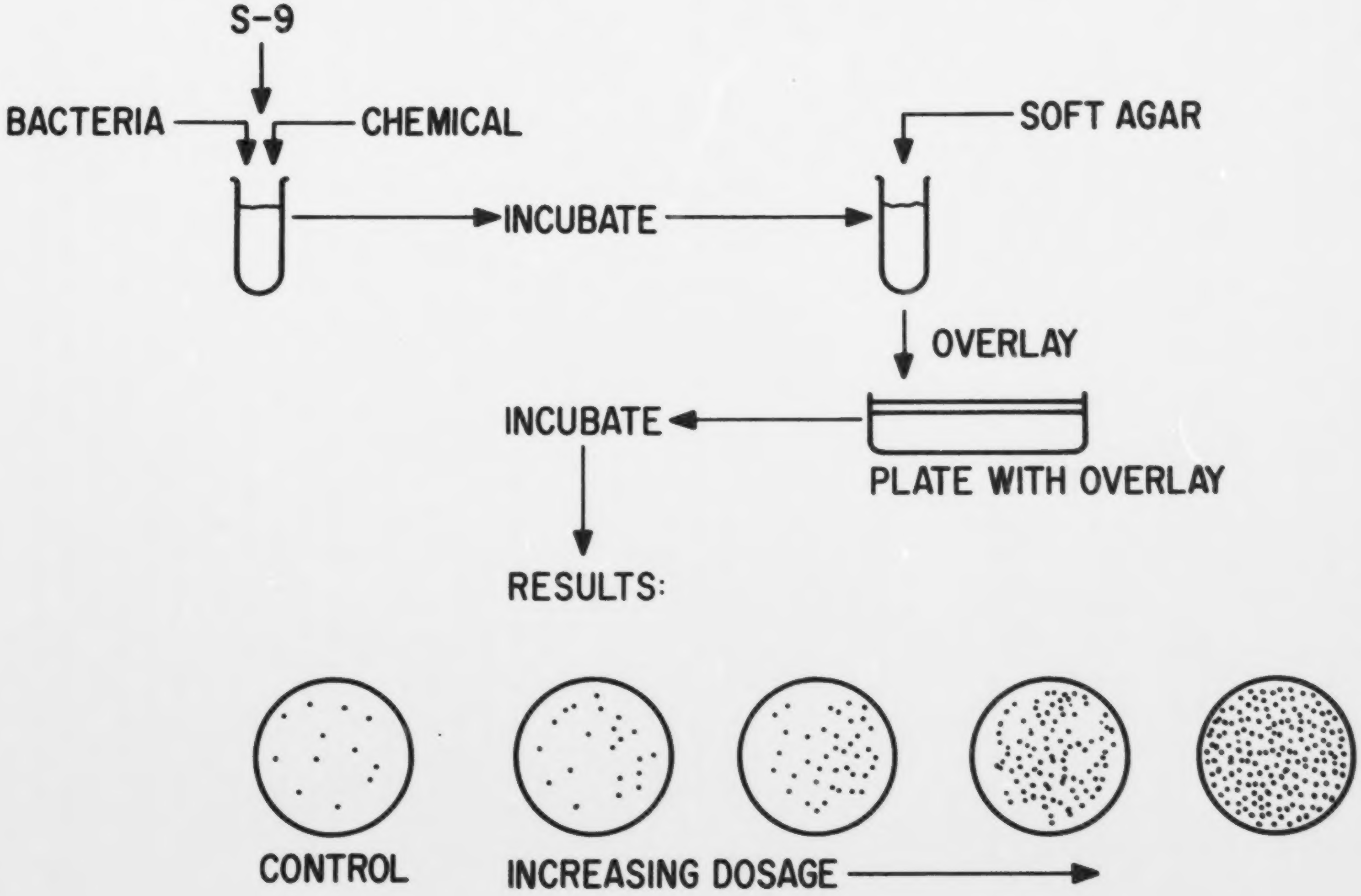
S-9
BACTERIA
CHEMICAL
INCUBATE
SOFT AGAR
OVERLAY
INCUBATE
PLATE WITH OVERLAY
RESULTS:
CONTROL
INCREASING DOSAGE

Figure 4. Schematic diagram of the preincubation test.

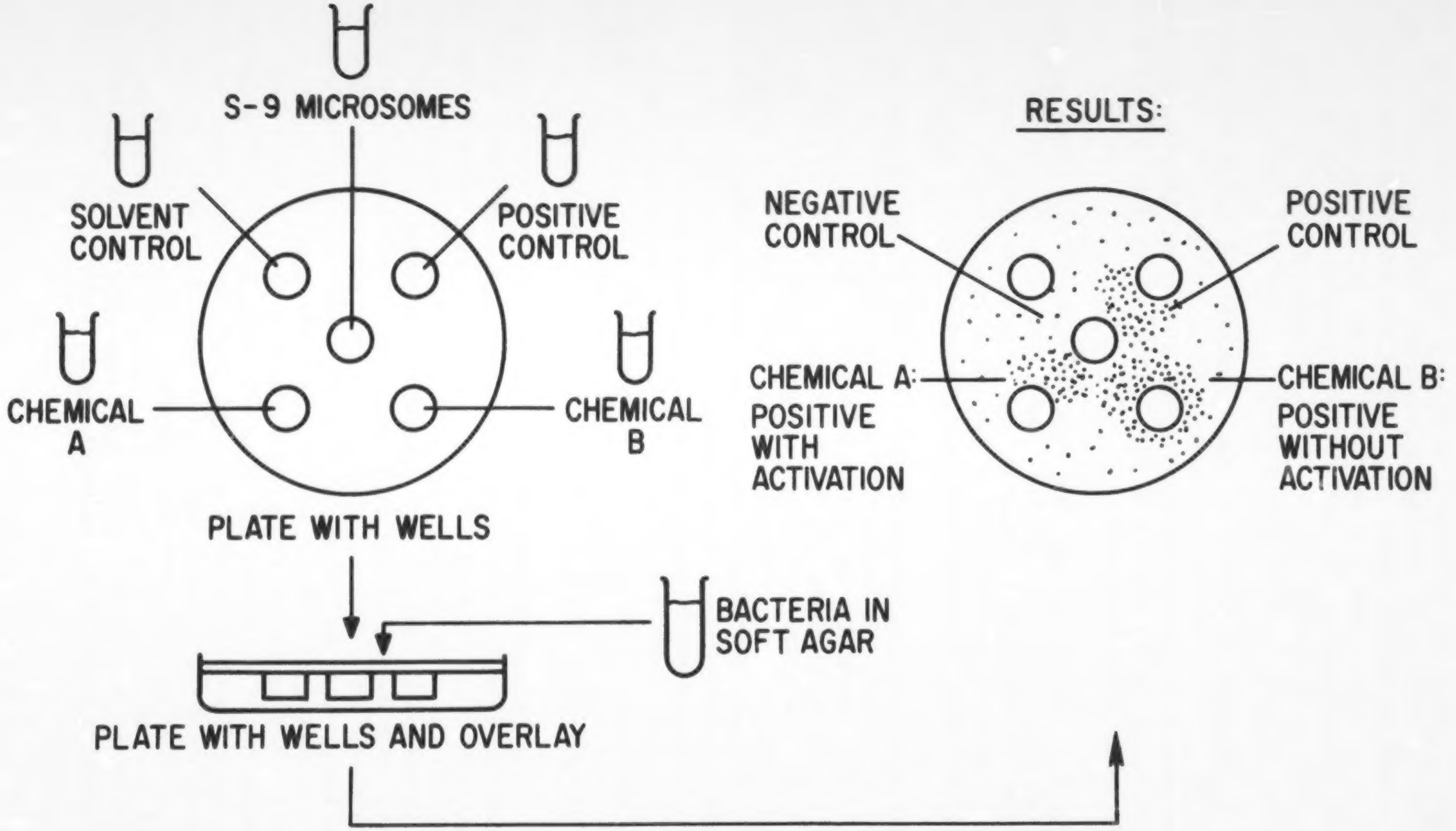
S-9 MICROSOMES
SOLVENT CONTROL
POSITIVE CONTROL
CHEMICAL A
CHEMICAL B
PLATE WITH WELLS
BACTERIA IN SOFT AGAR
PLATE WITH WELLS AND OVERLAY
RESULTS:
NEGATIVE CONTROL
POSITIVE CONTROL
CHEMICAL A: POSITIVE WITH ACTIVATION
CHEMICAL B: POSITIVE WITHOUT ACTIVATION

Figure 5. Schematic diagram of the well test.

second question is this: Water is an essential medium that supports life. We started by chemical analysis, we've gone to toxicity of aquatic organisms. Now there are organisms that live in air. Next year are we going to be analyzing the effect of toxicity of air on the variety of organisms such as butterfiles, mosquitos, sparrows, etc.? Maybe you gentlemen are missing a big bet. I would appreciate a reply to both of these questions.

Answer: I'm not sure I picked up the question. I heard the comment.

Question: You know what we are talking about as far as BAT is concerned. Are you going to spring an air problem on us just as you sprung the water problem on us?

Answer: The main reason we had the session now and sponsored a session about two years ago called "Industry Takes Initiative" was to encourage industry to take a look at the processes and to avoid as much as possible end of the line treatment by looking at process modification and changing the way things are made.

You're saying you know what may come out in BAT or what may come out of guidelines in Washington. But we're trying to say that, although there may be some numbers that come out of the guidelines in Washington, the national numbers will not cover everything. The specific problems at each facility must be considered individually. For instance, at a steel plant, the general type of toxicants is known - such as polynuclear hydrocarbons from a coke operation. In fact, Congress and our agency are already changing the requirements in many industries where they have, you might say, backed off in the BAT area and have gone to Best Conventional Treatment. And I agree with them because they are eliminating the need to install expensive pollution control equipment that really has little effect for the cost.

The purpose of this session is to get you, the industry, to start considering the toxicity testing described here today. If you look at processes in your own plant and current treatment technology, then combine that with some toxicity testing, you can determine and install the treatment technology that will eliminate some as yet unregulated toxicants in addition to "standard" toxicants and put yourself ahead of the game. But you must first evaluate your individual process to predict future requirements. So I think that's an answer to the first question as far as that goes. And as far as the second question goes, I don't know about measuring butterflies, but we are in fact significantly concerned about air emissions to the extent that some get into the water. Now in your facility I don't think that there would be that much of a problem, but say for instance, municipal sludge treatment and incineration is a beautiful example of delaying the introduction of a toxicant to the receiving waters. By incinerating a toxicant removed at a treatment plant, you may just volatilize it so it can fall back into the water. It's estimated, I think, that 80 to 90 percent of the PCBs going into Lake Erie and Lake Michigan are from the air not from the water. You need to look at what you're doing with your waste, and if you're incinerating something, are you really destroying it or are you only volatilizing it? And so you may need to be concerned about air emissions. And certainly the RCRA requirements I think fit very strongly into this.

Question: Excuse me. I want to go back to that. He says that we should look into it but back with Mr. Macek - he didn't say how frequently we had to test. Like with your wastewater system, how often do we have to run a bioassay? Daily, weekly, monthly, bimonthly, are you going to set up some sort of system?

Answer: There have been several people here today who have said you cannot set a time and say if you sample it every day or every week or every month that you will thereby resolve your problem. You're going to have to know, you're going to have to learn what your system is and learn how frequently you have to test in order to get the answer. In some systems it may turn out that there is just no change over any reasonable amount of time, so you could sample on a very infrequent basis. But there are other systems that change on an hourly basis, so there is really no way you can set up a sampling program whereby you sample infrequently and not miss certain things that will happen. You would have to have almost a continuous test in some cases or find some other way to measure to tie it down.

Answer: Just to further clarify. It would be very easy to come out and say that every industry will run a 24-hour test. Every industry will run a 96-hour test. And, this regulatory-wise, would be much easier. Maybe that's what you all wanted to hear us say today. But we're trying to say, which is I think a little more difficult for both of us, that we and the states want to work with you and for you to go ahead on your own to look at your specific facility. You may have only one product line, but we have plants where there are several thousand products in one plant. We would much rather work with you to try to gear the requirements or gear the testing to your specific operation, or have the states work with you if you're going ahead on your own, or have your consultant work with you. We're not trying to avoid setting numbers; it's just that there's so much variability from one type of industry to another that I think it would be unfair to you for us to propose to the region an absolute set of guidelines. And so I hope that we're not sounding like we're putting you off by failing to give you specific numbers without going to a particular class.

Answer: What Ken's saying is we're taking the hard way. It is much more difficult from our standpoint to work to try to sit down and figure out which tests should be run on what frequency, at what facility and how to use the results than it is to say every discharger in the Region shall have to run one 96-hour test or one 24-hour test on 10 percent effluent or 100 percent effluent and if he gets this score he is thereby failed or passed. That is an easy way - an easy approach which does not yield an answer. And we just don't need any more nonanswers.

RAPID ASSESSMENT METHODS (FISH COUGH RESPONSE, ETC.)

Robert Drummond
U.S. EPA, Environmental Research Laboratory
Duluth, Minnesota

Rapid assessment methods are not a new thing as people have been researching this area for several years. For example, there is an EPA publication called *Short-Term Tests for Health and Ecological Effects*. A number of tests are outlined in this publication such as fresh water algal assay bottle tests; acute fish toxicity tests; subchronic embryo-larval tests; chronic fish toxicity tests with flag fish; fish avoidance tests; acute invertebrate toxicity tests; subchronic invertebrate toxicity tests; acute plant toxicity tests; and acute static and acute flow-through tests with marine fish. Some of the endpoints of these tests, their strengths, weaknesses, status of development, application, and some idea of the cost of the test and people to contact are also discussed.

Other recent publications are concerned primarily with fish as a tool for biomonitoring effluents. Some of these papers are: *A Computerized System for Monitoring Fish Activity* (Virginia Polytechnic Institute, Virginia); *A Continued Biological Monitoring System to Predict Chronic Effects of Toxicants* (Virginia Polytechnic Institute, Virginia); *Fish Locomotor Behavior Patterns as a Monitoring Tool* (Morgan, South Africa); *Fish Activity Monitors* (Water Research Center, England); *Ventilatory Patterns of Fish Using a Microcomputer Monitoring System* (U.S. Army Medical Center, Maryland); *An Automated Biological Monitoring Facility for Rapid Assessment of Industrial Effluents* (Virginia Polytechnic Institute, Virginia); *Bluegill Respiratory Activity and Prediction of Chronic Toxicity* (Proctor and Gamble, Cincinnati, Ohio); and *Biomonitoring with Fish: An Aid to Industrial Effluent and Surface Water Quality Control* (Morgan, South Africa). Our particular laboratory (U.S. EPA, Environmental Research Laboratory-Duluth), has been involved in studies that are summarized in publications titled *Procedures for Measuring Cough Rates of Fish* and *The Fish Cough Response: A Method for Evaluating the Quality of Treated Complex Effluents*.

In order for behavioral responses of fish to be useful for biomonitoring effluents the test procedure must be inexpensive, simple enough to be carried out by non-technical personnel in a short period of time, and the endpoint being measured must have some relevance to the animal's ability to live and function properly. Of the various behavioral endpoints being considered, five appear to be closely allied to the animal's well-being and meet these other criteria. They are: 1) reproductive habits, 2) feeding patterns/habits, 3) fear responses, 4) the righting reflex, and 5) respiration. In a further breakdown, respiratory tests, in my opinion, are the most applicable and advantageous as an endpoint for monitoring effluents for a variety of reasons.

The term respiration as used here is an overall term denoting gill purge activity (cough rates), ventilation rate/amplitude, oxygen consumption, or any combination of the above. Advantages of using fish respiration as an endpoint become clear when one reviews the abundance of literature that has been published showing that numerous toxicants/effluents affect fish respiration rates. Fish act as an integrator of toxicity - synergistic or antagonistic effects of an effluent will be reflected in terms of changes in respiration rates. These changes occur within minutes or hours at lethal concentrations. At sublethal concentrations it may take 1 to 3 days for a change to appear. Thus, the response appears quickly. Another advantage is that the response is graded to the concentration. The intensity of the response can be used as a predictor of adverse effects. Lastly, the test is adaptable to both flow-through and recirculating bioassays. The recirculating bioassay approach has a big advantage in that hundreds of effluent samples (5 to 8 gallons) can be shipped to a central point for testing. The small volume of effluent required and speed at which respiratory changes appear make this an attractive approach for screening out those effluents that are of concern and need further testing.

We are now at a point where it is no longer necessary to observe fish directly to tabulate changes in respiratory rates. These data can be collected, tabulated, and analyzed automatically using computers or other automatic data processing devices. The automated approach allows more data to be collected and processed accurately at an affordable cost.

TEST ORGANISM ACQUISITION AND CULTURING IN THE LAB

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U.S. EPA, Region V
Chicago, Illinois

Good afternoon. The purpose of this discussion is to cover some of the major considerations for culturing or acquiring organisms. For this discussion, I'll use the working definitions that culturing organisms is the rearing of these test organisms for the entire lifecycle, that is, the entire reproductive cycle within the lab, while acquiring organisms is receiving them from an outside source and holding within the lab for a duration of time. The purpose of both techniques is to provide test organisms in which as many variables as possible are controlled with the ideal situation being that the only variable between the organisms is the test variable. The achievable goal, on the other hand, is to provide test organisms which are healthy, relatively free of pollutants, of known age, and which are physiologically representative of the species. Two of the general points to consider in culturing and holding organisms are that the holding or rearing facilities should be of a size which is adequate for the number and species of organism being utilized and the water supply to the culture unit should be of high quality and be delivered through inert piping. This is not to say that one must have a pristine water source for a culture unit. There are successful units now operating which use deep well water, river water, lake water, and even chlorinated tap water.

Many factors produce stress in organisms in the lab. Stress-producing factors should be avoided. The organisms should be shielded from any excess noise. The loading capacity of the facility should not be exceeded, and proper diet should be maintained at all times. Also, one must consider that for some organisms special environmental requirements are necessary such as flowing water or fluctuating water levels.

As was mentioned earlier this morning, the recommended construction materials for toxicity testing are teflon, glass, and number 316 stainless steel. Fortunately, holding facilities are not so limited in the choice of materials. For many years, concrete raceways and troughs were used for holding fish within the lab. These facilities have the disadvantage that they are permanent and do not allow flexibility within the lab. Recently, however, fiberglass tanks are more in line with what is needed because they are movable, durable, and versatile. And additionally, these units can be rigged to be self-cleaning, which is a real plus for anyone that has to work with the animals.

Table 1 is a list of the recommended test species and test temperatures. It includes also the salt water organisms, which I am not addressing. I would like to say that there are occasions when an organism would be used for a test that is not included in this list. In the case of an effluent toxicity test sometimes it may be desirable to use a sensitive species that is indigenous to the receiving water and/or is commercially or recreationally important to the receiving strain.

Let us now move to the test organisms themselves. The test organisms can be broken into two groups: the fish and the invertebrates. Fish may be obtained from three different sources. They may either be reared in the lab, obtained from a hatchery, or they may be collected from the wild population.

Table 1

RECOMMENDED SPECIES AND TEST TEMPERATURES

Species	Test Temperature (°C)[a]
Freshwater	
Vertebrates	
Coho salmon, *Oncorhynchus kisutch*	12
Rainbow trout, *Salmo gairdneri*	12
Brook trout, *Salvelinus fontinalis*	12
Goldfish, *Carassius auratus*	22
Fathead minnow, *Pimephales promelas*	22
Channel catfish, *Ictalurus punctatus*	22
Bluegill, *Lepomis macrochirus*	22
Invertebrates[a]	
Daphnids, *Daphnia magna* or *D. pulex*	17
Amphipods, *Gammarus lacustris*, *G. fasciatus*,	17
or *G. pseudolimnaeus*	17
Crayfish, *Orconectes* sp., *Cambarus* sp., *Procambarus*	22
sp., or *Pacifastacus leniusculus*	22
Stoneflies, *Pteronarcys* sp.	12
Mayflies, *Baetis* sp. or *Ephemerella* sp. *Hexagenia*	17
limbata or *H. bilineata*	22
Midges, *Chironomus* sp.	22

Table 1

RECOMMENDED SPECIES AND TEST TEMPERATURES (Continued)

Species	Test Temperature (°C)[a]
Marine and Estuarine	
Vertebrates	
Sheepshead minnow, Cyprinodon variegatus	22
Mummichog, Fundulus heteroclitus	22
Longnose killifish, Fundulus similis	22
Silverside, Menidia sp.	22
Threespine stickleback, Casterosteus aculeatus	22
Pinfish, Lagodon rhomboides	22
Spot, Leiostomus xanthurus	22
Shiner perch, Cymatogaster aggregata	12
Pacific staghorn sculpin, Leptocottus armatus	12
Sanddab, Citharichthys stigmaeus	12
Flounder, Paralichthys dentatus, P. lethostigma	22
English sole, Parophrys vetulus	12
Marine and estuarine	
Invertebrates[a]	
Shrimp, Penaeus setiferus, P. duorarum, or P. aztecus	22
Grass shrimp, Palaemonetes sp.	22
Shrimp, Crangon sp.	22
Oceanic shrimp, Pandalus jordani	12
Blue crab, Callinectes sapidus	22
Dungeness crab, Cancer magister	12
Mysid shrimp, Mysidopsis sp., Neomysis sp.	22
Atlantic oyster, Crassostrea virginica	22
Pacific oyster, Crassostrea gigas	20

[a] Freshwater amphipods, daphnids, and midge larvae and shrimp should be cultured and tested at the recommended test temperature. Other invertebrates should be held and tested within 5°C of the temperature of the water from which they were obtained. If the recommended test temperature is not within this range, they should be tested at the temperature from the series 7, 12, 17, 22, and 27°C that is closest to the recommended test temperature and is within the allowed range.

Material obtained from: Methods for Measuring the Acute Toxicity of Effluents to Aquatic Organisms. U.S. Environmental Protection Agency, 1978.

I'd like to address the collection from the wild population first. As was mentioned this morning, I would put collection from the wild population as the last resort in the case of fish, for the reasons that the life history is not known, the fish population may be diseased, the population may be hybridized, or it may already be contaminated with pollutants. Fish reared in the laboratory, on the other hand, present a real source and a continuous source of fish for most laboratories. As I stated earlier, the water supply should be of high quality, but is not limited to a source such as a deep well. An example is our own laboratory; our only source of water is chlorinated tap water supply. What we did was take the hot and cold water taps, put on a mixing valve to regulate the temperature and send the water through an activated carbon filter. The carbon filter is capable of removing the total residual chlorine down to less than five parts per billion and the unit itself has a capacity of 5,000 gallons a day, which is a lot more than most of you would ever anticipate using.

The advantages to maintaining a culture are many. As I mentioned earlier, it provides a continuous supply of fish of all stages. This includes eggs and allows for testing of the more sensitive life stages, a topic which was covered earlier. The life history of the fish from a culture unit is documented, and the fish remain relatively stress-free. But I think the most important aspect of having a rearing unit is that the fish are maintained in a disease-free state. As some of you well know, there is nothing more frustrating than starting a bioassay and having your control fish start dying off because of a disease problem. However, along with the advantages of the culture unit, there are some disadvantages. I feel that these are minor and probably are far outweighed by the advantages. The first disadvantage is that there is a moderate initial cost for setting up a culture unit. It would be hard for me to describe the entire unit we've got at the lab, but before we moved to our new facility, we set up an entire culture unit in a room for about $700. And the second disadvantage of the culture unit is that there is a need for continuous manpower supply to maintain the organisms.

The second source of fish I mentioned was hatchery fish. I would suggest that if you start looking for hatchery fish, one thing to consider is that the hatchery selected should be one which promotes the inbreeding of the parent stock to maintain the stock, rather than to replenish the parent stock from an outside source. Therefore I believe a hatchery should be picked in which the hatchery fish are several generations removed from the wild population to assure a better supply of test organisms.

There are some advantages to receiving fish from a hatchery. One, a lot of you may be limited in space, and a rearing unit does take up some space. But by the same token, a holding unit will also take up some floor space. Usually one can receive more species of fish from a hatchery than one can rear in the lab. The second advantage over a culture unit is that there is less time spent in maintaining the stock. You only need someone to work on the fish when you have them in the lab. There are several disadvantages to receiving hatchery fish. The desired size or age of fish that you may want may not be available at the time you need to do your testing. Transport from the hatchery to the lab causes stress on the organisms, and also there has to be some arrangement made for transport of the fish. Sometimes long-term planning is required to allow the hatchery to know the species and the number of fish that will be needed. But I think the most important point here is that fish received from an outside source have the potential to be diseased. Therefore, hatchery-reared fish must first be quarantined at least 10 days, preferably 21, before used as test organisms. Additionally, it may be advisable to give the fish a prophylactic disease treatment upon arrival in the lab. At this point I'd like to comment that on the disease treatment in hatchery fish, rather the disease prevention of fish received from an outside source. Table 2 includes the recommended prophylactic and therapeutic disease treatments, and Figure 1 is a copy of the procedure we use at Central Regional Lab. And I'd like to emphasize that any fish that we receive from an outside source are given a prophylactic disease treatment.

One thing I tried to do was find some sources of hatchery fish. Commonly, we go to federal and state hatcheries. Unfortunately, these facilities are not available to everyone in the audience. And so, in digging around, I did find some information. One possible contact for getting test fish would be the American Fish Farmer Federation. Their administrative offices are in Lonok, Arkansas, and they can put you in touch with bait dealers in your area. Another source may be to obtain a copy of the Annual Buyer's Guide published by the magazine Commercial Fish Farmer where they have listed all their members under the type of services provided.

The maintenance of any fish for testing should follow the strictest quality control practices and a log should be kept on all the aspects of rearing or maintaining these test organisms. I had thought about talking about the actual maintenance practices, but I don't think that time would allow me to cover all the points. I took Table 3 from a publication by the Sport Fish and Wildlife Service that showed the organic contamination of fish food. This is something that should be considered in your background information.

Table 2

RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH[a]

Disease	Chemical	Concentration (mg/1)	Duration of[c] Treatment
External Bacteria	Oxytetracycline hydrochloride (water soluble)	25[b]	30-60 min
	Procaine Penicillin G in Dihydrostreptomycin sulfate solution (Franklin Lab, Denver, CO)	(3ml/100 gal)	48-72 hrs
	Benzalkonium chloride (HYAMINE 1622R)	1-2[b]	30-60 min
	Nitrofurazone (water mix)	3-5[b]	30-60 min
	Neomycin sulfate	25	30-60 min
Monogenetic trematodes fungi, and external protozoa[d]	Formalin *plus* zinc-free malachite green oxalate	25 0.1	1-2 hrs
	Formalin	150-250	30-60 min
	Potassium permanganate	2-6	30-60 min
	Sodium chloride	15000-30000 2000-4000	5-10 min dip (e)
	DEXONR (35% Active Ingredient)	20	30-60 min
Parasitic copepods	Trichlorfon (MASOTENR)	0.25[b]	Continuous[f]

[a] This table indicates the order of preference of treatments that have been reported to be effective, but their efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Caution: test treatments on small lots of fish before making large-scale applications. Fish should not be treated the first day they are in the facility.

Material obtained from: Methods for Measuring the Acute Toxicity of Effluents to Aquatic Organisms. U.S. Environmental Protection Agency, 1978.

C. DISEASE PREVENTION TREATMENT.

All fish shall be given preventive treatment for disease or parasites upon receipt from the hatchery. Treat the fish in the holding tank in the following day-by-day sequence and do not stop aeration at any time.

Day 1: Two to three days before the fish arrive, fill the holding tank with nonchlorinated water to the 5-gallon level and mark the level permanently with waterproof marker. Add 45 more gallons of nonchlorinated water and mark the 50-gallon level. Fill three storage containers with nonchlorinated water and bring to room temperature. Aerate the water continuously. (Caution: Be sure to dechlorinate with a carbon filter if water contains chlorine).

Day 3: Fish arrival. Without opening, float the plastic shipping bag (water and fish) in the tank and acclimate to tank temperature for 1 - 2 hours before releasing the fish to tank water.

Day 4: (a) Add 5 grams ACS grade potassium permanganate ($KMnO_4$) to 450 milliliters distilled or deionized water. When dissolved dilute up to 500 milliliters. This is a 1% solution of $KMnO_4$. This solution is used on days 4 and 5, and any remaining solution is then discarded.

(b) Add 190 milliliters of the $KMnO_4$ solution to the holding tank containing 50 gallons of water. (Caution: Stir while adding the $MNnO_4$ to achieve a uniform solution.) Hold the fish in the $KMnO_4$ solution for 1/2 hour. Do not exceed the 1/2-hour limit, but continue the treatment to that time even if the fish begin to surface or otherwise seem stressed.

(c) After 25 minutes of treatment, begin to drain the holding tank to the 5-gallon mark. At 30 minutes add water from the storage container to bring the tank volume to 50 gallons.

(d) Add to the holding tank 1 - 2 milliliters of 0.1 N sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) solution, or until the water turns yellow-brown. (To prepare 0.1 N sodium thiosulfate solution dissolve 25 grams sodium thiosulfate in volumetric flask and dilute to 1 liter with distilled or deionized water.) Color change is slow, so do not be concerned if the water does not become yellow-brown immediately. Refill storage container with non-chlorinated water. Be sure water is at room temperature before it is used on day 5.

Figure 1. Disease-prevention procedure used at Central Regional Laboratory, U.S. EPA Region V, Chicago.

Day 5: Repeat steps (b), (c), and (d) from day 4. It is not necessary to refill the storage containers on this day.

Day 6: (a) Add 4,750 milligrams of tetracycline (the material from nineteen 250-milligram capsules) to 500 milliliters of warm tap water. Shake until dissolved. Pour this solution into the holding tank and mix gently. Leave the fish in this solution. Fish are fed for the first time on this day.

(b) Fill storage containers with effluent-receiving water for acclimation (Section II, D).

Day 7: Feed fish and clean bottom of tank with siphon. Aerate water.

Material obtained from: ORSANCO 24-Hour Bioassay. Ohio River Water Sanitation Commission, 1974.

Figure 1. Disease-prevention procedure used at Central Regional Laboratory, U.S. EPA Region V, Chicago (continued).

Table 3
SELECTED CONTAMINANTS DETECTED IN DIETS ANALYZED INTERMITTENTLY
AT FPRL FROM AUGUST, 1972 - AUGUST, 1973

	Residues (ug/g)						
Diets	DDT[a]	PCB[b]	Hexchlorobenzene (HCB)	Dieldrin	Endrin	Total Organochlorine Content	Phthalate[c] esters
Oregon Moist	0.06	0.30	-[d]	-	-	0.36	-
Glenco	0.11	0.30	-	-	-	0.41	-
Clark	0.11	0.20	-	-	-	0.31	-
Silver Cup (2 samples)	0.08- 0.17	0.20- 0.32	- 0.06	- 0.01	- -	0.37 0.555	- -
EWOS (3 samples)	0.15- 0.39	0.20- 0.30	0.008- 0.046	0.01- 0.02	- 0.01	0.361- 0.766	- 3.0
Colorado State Diets (9 samples)	0.11- 0.84	0.10- 2.80	- 0.003	0.01- 0.30	- 0.01	0.213- 3.953	- -
BSFW Hatchery Diets (2 samples)	0.12- 0.19	0.20- 0.30	-	- 0.01	-	0.32- 0.50	- -
Purina Catfish Chow	-	-	-	0.01	-	0.01	-
Reference Research Diet (4 samples)	-	<0.1	-	-	-	<0.1	-
Minimum Detection Limits	0.005	0.1	0.0001	0.01	0.01		0.1- 0.5

[a] All DDT analogs.
[b] All polychlorinated biphenyls, but usually Aroclor^R 1254 and 1260.
[c] Di-n-butyl phthalate and di-2-ethylhexyl phthalate.
[d] None detected.

Table Obtained From: Acquisition and Culture of Research Fish: Rainbow Trout, Fathead Minnows, Channel Catfish and Bluegills. U.S. Bureau of Sport Fisheries and Wildlife, 1975.

The last topic that I was going to talk on was invertebrates. And I saved this for last because there's not much information I can give you. With the exception of Daphnia, invertebrates are difficult to maintain in the laboratory for any extended length of time because they have very precise environmental requirements. For the most part, individuals seeking these invertebrates are required to develop their own sources - that means going out and scouring the streams in your area. The reason for this is that, for the most part, invertebrates do not ship well, and it's hard to maintain good quality up until the test time. The exception in the invertebrate field are the Daphnia. Daphnia can be obtained from many sources. If one is looking to start his own Daphnia culture, small numbers of animals can be obtained from several biological supply houses. In most cases it's best to let the people know that you are looking for a particular species and what they are to be used for so that you can guarantee proper identification. On the other hand, if one is looking for large numbers of Daphnia, I know of at least one supply facility in Minneapolis, Minnesota, Aquatic Life, that shipped units of Daphnia between 12,000 and 15,000 in number for around $50, including the air freight.

In summary, I would like to say that this has been a general discussion on rearing and holding, and I would reemphasize that good quality control practices are needed to maintain the test organisms. If this is done, it makes the whole job of running bioassay tests a lot simpler.